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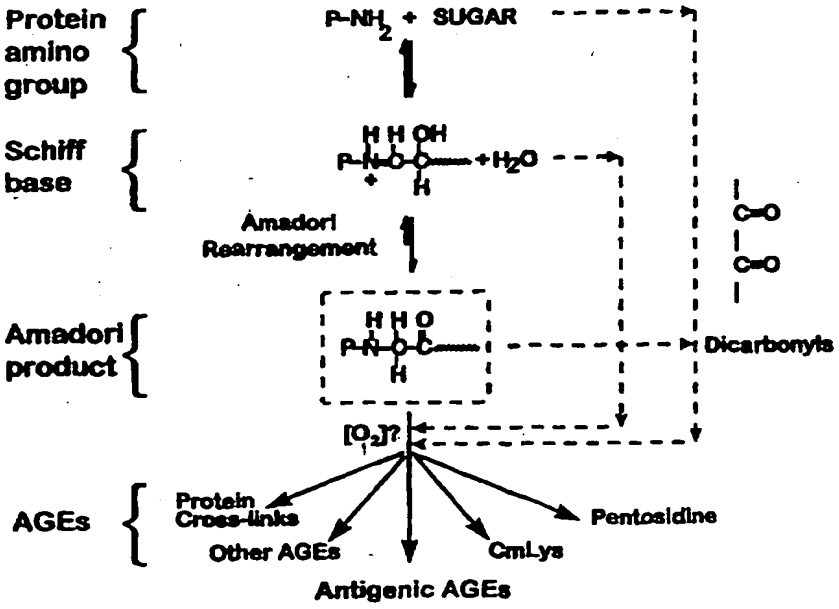

(57) Abstract

The instant invention provides compositions and methods for modeling post-Amadori AGE formation and the identification and characterization of effective inhibitors of post-Amadori AGE formation, and such identified inhibitor compositions.

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Title: Advanced Glycation End-product Intermediaries and Post-Amadori Inhibition

This application claims priority to U.S. Provisional Application for Patent serial number 60/003,268, filed September 12, 1995, the contents of which are hereby incorporated in the entirety.

Statement of Government Rights

Some of the work disclosed has been supported in part by NIH Grant DK 43507, therefore, the United States Government may have certain rights in the invention..

BACKGROUND OF THE INVENTION

The instant invention is in the field of Advanced Glycation End-products (AGEs), their formation, detection, identification, inhibition, and inhibitors thereof.

Protein Aging and Advanced Glycosylation End-products

Nonenzymatic glycation by glucose and other reducing sugars is an important post-translational modification of proteins that has been increasingly implicated in diverse pathologies. Irreversible nonenzymatic glycation and crosslinking through a slow, glucose-induced process may mediate many of the complications associated with diabetes. Chronic hyperglycemia associated with diabetes can cause chronic tissue damage which can lead to complications such as retinopathy, nephropathy, and atherosclerotic disease. (Cohen and Ziyadeh, 1996, *J. Amer. Soc. Nephrol.* **7**:183-190). It has been shown that the resulting chronic tissue damage associated with long-term diabetes mellitus arise in part from *in situ* immune complex formation by accumulated immunoglobulins and/or antigens bound to long-lived structural proteins that have undergone Advanced Glycosylation End-product (AGE) formation, via non-enzymatic glycosylation (Brownlee et al., 1983, *J. Exp. Med.* **158**:1739-1744). The primary protein target is thought to

be extra-cellular matrix associated collagen. Nonenzymatic glycation of proteins, lipids, and nucleic acids may play an important role in the natural processes of aging. Recently protein glycation has been associated with ß-amyloid deposits and formation of neurofibrillary tangles in Alzheimer disease, and possibly other neurodegenerative diseases involving amyloidosis (Colaco and Harrington, 1994, *NeuroReport* **5**: 859-861). Glycated proteins have also been shown to be toxic, antigenic, and capable of triggering cellular injury responses after uptake by specific cellular receptors (see for example, Vlassara, Bucala & Striker, 1994, *Lab. Invest.* **70**:138-151; Vlassara et al., 1994, *PNAS(USA)* **91**:11704-11708; Daniels & Hauser, 1992, *Diabetes* **41**:1415-1421; Brownlee, 1994, *Diabetes* **43**:836-841; Cohen et al., 1994, *Kidney Int.* **45**:1673-1679; Brett et al., 1993, *Am. J. Path.* **143**:1699-1712; and Yan et al., 1994, *PNAS(USA)* **91**:7787-7791).

The appearance of brown pigments during the cooking of food is a universally recognized phenomenon, the chemistry of which was first described by Maillard in 1912, and which has subsequently led to research into the concept of protein aging. It is known that stored and heat-treated foods undergo nonenzymatic browning that is characterized by crosslinked proteins which decreases their bioavailibility. It was found that this Maillard reaction occurred *in vivo* as well, when it was found that a glucose was attached via an Amadori rearrangement to the amino-terminal of the α-chain of hemoglobin.

The instant disclosure teaches previously unknown, and unpredicted mechanism of formation of post-Amadori advanced glycation end products (Maillard products; AGEs) and methods for identifying and characterizing effective inhibitors of post-Amadori AGE formation. The instant disclosure demonstrates the unique isolation and kinetic characterization of a reactive protein intermediate competent in forming post-Amadori AGEs, and for the first time teaching methods which allow for the specific

elucidation and rapid quantitative kinetic study of "late" stages of the protein glycation reaction.

In contrast to such "late" AGE formation, the "early" steps of the glycation reaction have been relatively well characterized and identified for several proteins (Harding, 1985, *Adv. Protein Chem.* **37**:248-334; Monnier & Baynes eds., 1989, *The Maillard Reaction in Aging, Diabetes, and Nutrition* (Alan R. Liss, New York); Finot et al., 1990, eds. *The Maillard Reaction in Food Processing, Human Nutrition and Physiology* (Birkhauser Verlag, Basel)). Glycation reactions are known to be initiated by reversible Schiff-base (aldimine or ketimine) addition reactions with lysine side-chain ε-amino and terminal α-amino groups, followed by essentially irreversible Amadori rearrangements to yield ketoamine products e.g. 1-amino-1-deoxy-ketoses from the reaction of aldoses (Baynes et al., 1989, in The Maillard Reaction in Aging, Diabetes, and Nutrition, ed. Monnier and Baynes, (Alan R. Liss, New York, pp 43-67). Typically, sugars initially react in their open-chain (not the predominant pyranose and furanose structures) aldehydo or keto forms with lysine side chain ε-amino and terminal α-amino groups through reversible Schiff base condensation (Scheme I). The resulting aldimine or ketimine products then undergo Amadori rearrangements to give ketoamine Amadori products, i.e. 1-amino-1-deoxy-ketoses from the reaction of aldoses (Means & Chang, 1982, *Diabetes* **31, Suppl. 3**:1-4; Harding, 1985, *Adv. Protein Chem.* **37**:248-334). These Amadori products then undergo, over a period of weeks and months, slow and irreversible Maillard "browning" reactions, forming fluorescent and other products via rearrangement, dehydration, oxidative fragmentation, and cross-linking reactions. These post-Amadori reactions, (slow Maillard "browning" reactions), lead to poorly characterized Advanced Glycation End-products (AGEs).

As with Amadori and other glycation intermediaries, free glucose itself can undergo oxidative reactions that lead to the production of peroxide and highly reactive

fragments like the dicarbonyls glyoxal and glycoaldehyde. Thus the elucidation of the mechanism of formation of a variety of AGEs has been extremely complex since most *in vitro* studies have been carried out at extremely high sugar concentrations.

In contrast to the relatively well characterized formation of these "early" products, there has been a clear lack of understanding of the mechanisms of forming the "late" Maillard products produced in post-Amadori reactions, because of their heterogeneity, long reaction times, and complexity. The lack of detailed information about the chemistry of the "late" Maillard reaction stimulated research to identify fluorescent AGE chromophores derived from the reaction of glucose with amino groups of polypeptides. One such chromophore, 2-(2-furoyl)-4(5)-(2-furanyl)-1*H*-imidazole (FFI) was identified after nonenzymatic browning of bovine serum albumin and polylysine with glucose, and postulated to be representative of the chromophore present in the intact polypeptides. (Pongor et al., 1984, *PNAS(USA)* **81**:2684-2688). Later studies established FFI to be an artifact formed during acid hydrolysis for analysis.

A series of U.S. Patents have issued in the area of inhibition of protein glycosylation and cross-linking of protein sugar amines based upon the premise that the mechanism of such glycosylation and cross-linking occurs via saturated glycosylation and subsequent cross-linking of protein sugar amines via a single basic, and repeating reaction. These patents include U.S. Patents 4,665,192; 5,017,696; 4,758,853; 4,908,446; 4,983,604; 5,140,048; 5,130,337; 5,262,152; 5,130,324; 5,272,165; 5,221,683; 5,258,381; 5,106,877; 5,128,360; 5,100,919; 5,254,593; 5,137,916; 5,272,176; 5,175,192; 5,218,001; 5,238,963; 5,358,960; 5,318,982; and 5,334,617. (All U.S. Patents cited are hereby incorporated by reference in their entirety).

The focus of these U.S. Patents, are a method for inhibition of AGE formation focused on the carbonyl moiety

of the early glycosylation Amadori product, and in particular the most effective inhibition demonstrated teaches the use of exogenously administered aminoguanidine. The effectiveness of aminoguanidine as an inhibitor of AGE formation is currently being tested in clinical trials.

Inhibition of AGE formation has utility in the areas of, for example, food spoilage, animal protein aging, and personal hygiene such as combating the browning of teeth. Some notable, though quantitatively minor, advanced glycation end-products are pentosidine and N^{ε} -carboxymethyllysine (Sell and Monnier, 1989, *J. Biol. Chem.* **264**:21597-21602; Ahmed et al., 1986, *J. Biol. Chem.* **261**:4889-4894).

The Amadori intermediary product and subsequent post-Amadori AGE formation, as taught by the instant invention, is not fully inhibited by reaction with aminoguanidine. Thus, the formation of post-Amadori AGEs as taught by the instant disclosure occurs via an important and unique reaction pathway that has not been previously shown, or even previously been possible to demonstrate in isolation. It is a highly desirable goal to have an efficient and effective method for identifying and characterizing effective post-Amadori AGE inhibitors of this "late" reaction. By providing efficient screening methods and model systems, combinatorial chemistry can be employed to screen candidate compounds effectively, and thereby greatly reducing time, cost, and effort in the eventual validation of inhibitor compounds. It would be very useful to have *in vivo* methods for modeling and studying the effects of post-Amadori AGE formation which would then allow for the efficient characterization of effective inhibitors.

Inhibitory compounds that are biodegradeble and/or naturally metabolized are more desirable for use as therapeutics than highly reactive compounds which may have toxic side effects, such as aminoguanidine.

SUMMARY OF THE INVENTION

In accordance with the present invention, a stable post-Amadori advanced glycation end-product (AGE) precursor has been identified which can then be used to rapidly complete the post-Amadori conversion into post-Amadori AGEs. This stable product is a presumed sugar saturated Amadori/Schiff base product produced by the further reaction of the early stage protein/sugar Amadori product with more sugar. In a preferred embodiment, this post-Amadori/Schiff base intermediary has been generated by the reaction of target protein with ribose sugar.

The instant invention provides for a method of generating stable protein-sugar AGE formation intermediary precursors via a novel method of high sugar inhibition. In a preferred embodiment the sugar used is ribose.

The instant invention provides for a method for identifying an effective inhibitor of the formation of late Maillard products comprising: generating stable protein-sugar post-Amadori advanced glycation end-product intermediates by incubating a protein with sugar at a sufficient concentration and for sufficient length of time to generate stable post-Amadori AGE intermediates; contacting said stable protein-sugar post-Amadori advanced glycation end-product intermediates with an inhibitor candidate; identifying effective inhibition by monitoring the formation of post-Amadori AGEs after release of the stable protein-sugar post-Amadori advanced glycation end-product intermediates from sugar induced equilibrium. Appropriate sugars include, and are not limited to ribose, lyxose, xylose, arabinose, glucose, fructose, maltose, lactose, mannose, fructose and galactose. In a preferred embodiment the sugar used is ribose.

The instant invention teaches that an effective inhibitor of post-Amadori AGE formation via "late" reactions can be identified and characterized by the ability to inhibit the formation of post-Amadori AGE endproducts in an assay comprising; generating stable protein-sugar post-

Amadori advanced glycation end-product intermediates by incubating a protein with sugar at a sufficient concentration and for sufficient length of time to generate stable post-Amadori AGE intermediates; contacting said stable protein-sugar post-Amadori advanced glycation end-product intermediates with an inhibitor candidate; identifying effective inhibition by monitoring the formation of post-Amadori AGEs after release of the stable protein-sugar post-Amadori advanced glycation end-product intermediates from sugar induced equilibrium. In a preferred embodiment the assay uses ribose.

Thus the methods of the instant invention allow for the rapid screening of candidate post-Amadori AGE formation inhibitors for effectiveness, greatly reducing the cost and amount of work required for the development of effective small molecule inhibitors of post-Amadori AGE formation. The instant invention teaches that effective inhibitors of post-Amadori "late" reactions of AGE formation include derivatives of vitamin B_6 and vitamin B_1, in the preferred embodiment the specific species being pyridoxamine and thiamine pyrophosphate.

The instant invention teaches new methods for rapidly inducing diabetes like pathologies in rats comprising administering ribose to the subject animal. Further provided for is the use of identified inhibitors pyridoxamine and thiamine pyrophosphate *in vivo* to inhibit post-Amadori AGE induced pathologies.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a series of graphs depicting the effect of vitamin B_6 derivatives on AGE formation in bovine serum albumin (BSA). Figure 1A Pyridoxamine (PM); Figure 1B pyridoxal phosphate (PLP); Figure 1C pyridoxal (PL); Figure 1D pyridoxine (PN).

Figure 2 is a series of graphs depicting the effect of vitamin B_1 derivatives and aminoguanidine (AG) on AGE formation in bovine serum albumin. Figure 2A Thiamine

pyrophosphate (TPP); Figure 2B thiamine monophosphate (TP); Figure 2C thiamine (T); Figure 2D aminoguanidine (AG).

Figure 3 is a series of graphs depicting the effect of vitamin B_6 derivatives on AGE formation in human methemoglobin (Hb). Figure 3A Pyridoxamine (PM); Figure 3B pyridoxal phosphate (PLP); Figure 3C pyridoxal (PL); Figure 3D pyridoxine (PN).

Figure 4 is a series of graphs depicting the effect of vitamin B_1 derivatives and aminoguanidine (AG) on AGE formation in human methemoglobin. Figure 2A Thiamine pyrophosphate (TPP); Figure 2B thiamine monophosphate (TP); Figure 2C thiamine (T); Figure 2D aminoguanidine (AG).

Figure 5 is a bar graph comparison of the inhibition of the glycation of ribonuclease A by thiamine pyrophosphate (TPP), pyridoxamine (PM) and aminoguanidine (AG).

Figure 6A is a graph of the kinetics of glycation of RNase A (10 mg/mL) by ribose as monitored by ELISA. Figure 6B is a graph showing the dependence of reciprocal half-times on ribose concentration at pH 7.5.

Figure 7 are two graphs showing a comparison of uninterrupted and interrupted glycation of RNase by glucose (7B) and ribose (7A), as detected by ELISA.

Figure 8 are two graphs showing kinetics of pentosidine fluorescence (arbitrary units) increase during uninterrupted and interrupted ribose glycation of RNase. Figure 8A Uninterrupted glycation in the presence of 0.05 M ribose. Figure 8B Interrupted glycation after 8 and 24 hours of incubation.

Figure 9 is a graph which shows the kinetics of reactive intermediate buildup.

Figure 10 are graphs of Post-Amadori inhibition of AGE formation by ribose. Figure 10A graphs data where aliquots were diluted into inhibitor containing buffers at time 0. Figure 10B graphs data where samples were interrupted at 24h, and then diluted into inhibitor containing buffers.

Figure 11 is a graph showing dependence of the initial rate of formation of antigenic AGE on pH following

interruption of glycation.

Figure 12 are two graphs showing the effect of pH jump on ELISA detected AGE formation after interrupted glycation. Interrupted samples left 12 days at 37°C in pH 5.0 buffer produced substantial AGEs (33%; Figure 12 B) when pH was changed to 7.5, as compared to the normal control sample not exposed to low pH (Figure 12 A).

Figure 13 is a series of graphs depicting the effect of vitamin B_6 derivatives on AGE formation during uninterrupted glycation of ribonuclease A (RNase A) by ribose. Figure 13A Pyridoxamine (PM); Figure 13B pyridoxal-5'-phosphate (PLP); Figure 13C pyridoxal (PL); Figure 13D pyridoxine (PN).

Figure 14 is a series of graphs depicting the effect of vitamin B_1 derivatives and aminoguanidine (AG) on AGE formation during uninterrupted glycation of ribonuclease A (RNase A) by ribose. Figure 14A Thiamine pyrophosphate (TPP); Figure 14B thiamine monophosphate (TP); Figure 14C thiamine (T); Figure 14D aminoguanidine (AG).

Figure 15 is a series of graphs depicting the effect of vitamin B_6 derivatives on AGE formation during uninterrupted glycation of bovine serum albumin (BSA) by ribose. Figure 15A Pyridoxamine (PM); Figure 15B pyridoxal-5'-phosphate (PLP); Figure 15C pyridoxal (PL); Figure 15D pyridoxine (PN).

Figure 16 is a series of graphs depicting the effect of vitamin B_1 derivatives and aminoguanidine (AG) on AGE formation during uninterrupted glycation of bovine serum albumin (BSA) by ribose. Figure 16A Thiamine pyrophosphate (TPP); Figure 16B thiamine monophosphate (TP); Figure 16C thiamine (T); Figure 16D aminoguanidine (AG).

Figure 17 is a series of graphs depicting the effect of vitamin B_6 derivatives on AGE formation during uninterrupted glycation of human methemoglobin (Hb) by ribose. Figure 17A Pyridoxamine (PM); Figure 17B pyridoxal-5'-phosphate (PLP); Figure 17C pyridoxal (PL); Figure 17D pyridoxine (PN).

Figure 18 is a series of graphs depicting the effect of vitamin B_6 derivatives on post-Amadori AGE formation after

interrupted glycation by ribose. Figure 18A BSA and Pyridoxamine (PM); Figure 18B BSA and pyridoxal-5'-phosphate (PLP); Figure 18C BSA and pyridoxal (PL); Figure 18D RNase and pyridoxamine (PM).

Figure 19 are graphs depicting the effect of thiamine pyrophosphate on post-Amadori AGE formation after interrupted glycation by ribose. Figure 19A RNase, Figure 19B BSA.

Figure 20 are graphs depicting the effect of aminoguanidine on post-Amadori AGE formation after interrupted glycation by ribose. Figure 20A RNase, Figure 20B BSA.

Figure 21 is a graph depicting the effect of N^{α}-acetyl-L-lysine on post-Amadori AGE formation after interrupted glycation by ribose.

Figure 22 are bar graphs showing a comparison of post-Amadori inhibition of AGE formation by thiamine pyrophosphate (TPP), pyridoxamine (PM) and aminoguanidine (AG) after interrupted glycation of RNase (Figure 22A) and BSA (Figure 22B) by ribose.

Figure 23 is a bar graph showing the effects of Ribose treatment *in vivo* alone on rat tail-cuff blood pressure. Treatment was with 0.05 M, 0.30 M, and 1 M Ribose (R) injected for 1, 2 or 8 Days (D).

Figure 24 is a bar graph showing the effects of Ribose treatment *in vivo* alone on rat creatinine clearance (Clearance per 100 g Body Weight). Treatment was with 0.05 M, 0.30 M, and 1 M Ribose (R) injected for 1, 2 or 8 Days (D).

Figure 25 is a bar graph showing the effects of Ribose treatment *in vivo* alone on rat Albuminuria (Albumin effusion rate). Treatment was with 0.30 M, and 1 M Ribose (R) injected for 1, 2 or 8 Days (D).

Figure 26 is a bar graph showing the effects of inhibitor treatment *in vivo*, with or without ribose, on rat tail-cuff blood pressure. Treatment groups were: 25 mg/100 g body weight aminoguanidine (AG); 25 or 250 mg/100 g body

weight Pyridoxamine (P); 250 mg/100 g body weight Thiamine pyrophosphate (T), or with 1 M Ribose (R).

Figure 27 is a bar graph showing the effects of inhibitor treatment *in vivo*, with or without ribose, on rat creatinine clearance (Clearance per 100 g body weight). Treatment groups were: 25 mg/100 g body weight aminoguanidine (AG); 25 or 250 mg/100 g body weight Pyridoxamine (P); 250 mg/100 g body weight Thiamine pyrophosphate (T), or with 1 M Ribose (R).

Figure 28 is a bar graph showing the effects of inhibitor treatment *in vivo* without ribose, and ribose alone on rat Albuminuria (Albumin effusion rate). Treatment groups were: 25 mg/100 g body weight aminoguanidine (AG); 250 mg/100 g body weight Pyridoxamine (P); 250 mg/100 g body weight Thiamine pyrophosphate (T), or treatment with 1 M Ribose (R) for 8 days (D). Control group had no treatment.

Figure 29 is a bar graph showing the effects of inhibitor treatment *in vivo*, with 1 M ribose , on rat Albuminuria (Albumin effusion rate). Treatment groups were: 25 mg/100 g body weight aminoguanidine (AG); 25 and 250 mg/100 g body weight Pyridoxamine (P); 250 mg/100 g body weight Thiamine pyrophosphate (T), or treatment with 1 M Ribose (R) for 8 days (D) alone. Control group had no treatment.

Figure 30A depicts Scheme 1 showing a diagram of AGE formation from protein. Figure 30B depicts Scheme 2, a chemical structure of aminoguanidine. Figure 30C depicts Scheme 3, chemical structures for thiamine, thiamine-5'-phosphate, and thiamine pyrophosphate. Figure 30D depicts Scheme 4, chemical structures of pyridoxine, pyridoxamine, pyridoxal-5'-phosphate, and pyridoxal. Figure 30E depicts Scheme 5, kinetics representation of AGE formation. Figure 30F depicts Scheme 6, kinetics representation of AGE formation and intermediate formation.

Figure 31 shows a 125 MHz C-13 NMR Resonance spectrum of Riobonuclease Amadori Intermediate prepared by 24 HR reaction with 99% [2-C13]Ribose.

DETAILED DESCRIPTION

Animal Models for Protein Aging

Alloxan induced diabetic Lewis rats have been used as a model for protein aging to demonstrate the *in vivo* effectiveness of inhibitors of AGE formation. The correlation being demonstrated is between inhibition of late diabetes related pathology and effective inhibition of AGE formation (Brownlee, Cerami, and Vlassara, 1988, *New Eng. J. Med.* **318(20)**:1315-1321). Streptozotocin induction of diabetes in Lewis rats, New Zealand White rabbits with induced diabetes, and genetically diabetic BB/Worcester rats have also been utilized, as described in, for example, U.S. Patent 5,334,617 (incorporated by reference). A major problem with these model systems is the long time period required to demonstrate AGE related injury, and thus to test compounds for AGE inhibition. For example, 16 weeks of treatment was required for the rat studies described in U.S. Patent 5,334,617, and 12 weeks for the rabbit studies. Thus it would be highly desirable and useful to have a model system for AGE related diabetic pathology that will manifest in a shorter time period, allowing for more efficient and expeditious determination of AGE related injury and the effectiveness of inhibitors of post-Amadori AGE formation.

Antibodies to AGEs

An important tool for studying AGE formation is the use of polyclonal and monoclonal antibodies that are specific for AGEs elicited by the reaction of several sugars with a variety of target proteins. The antibodies are screened for resultant specificity for AGEs that is independent of the nature of the protein component of the AGE (Nakayama et al., 1989, *Biochem. Biophys. Res. Comm.* **162**: 740-745; Nakayama et al., 1991, *J. Immunol. Methods* **140**: 119-125; Horiuchi et al., 1991, *J. Biol. Chem.* **266**: 7329-7332; Araki et al., 1992, *J. Biol. Chem.* **267**: 10211-10214; Makita et al., 1992, *J. Biol. Chem.* **267**: 5133-5138). Such antibodies have been used to monitor AGE formation *in*

vivo and *in vitro*.

Thiamine - Vitamin B_1

The first member of the Vitamin B complex to be identified, thiamine is practically devoid of pharmacodynamic actions when given in usual therapeutic doses; and even large doses were not known to have any effects. Thiamine pyrophosphate is the physiologically active form of thiamine, and it functions mainly in carbohydrate metabolism as a coenzyme in the decarboxylation of α-keto acids. Tablets of thiamine hydrochloride are available in amounts ranging from 5 to 500 mg each. Thiamine hydrochloride injection solutions are available which contain 100 to 200 mg/ml.

For treating thiamine deficiency, intravenous doses of as high as 100 mg / L of parenteral fluid are commonly used, with the typical dose of 50 to 100 mg being administered. GI absorption of thiamine is believed to be limited to 8 to 15 mg per day, but may be exceed by oral administration in divided doses with food.

Repeated administration of glucose may precipitate thiamine deficiency in under nourished patients, and this has been noted during the correction of hyperglycemia.

Surprisingly, the instant invention has found, as shown by *in vitro* testing, that administration of thiamine pyrophosphate at levels above what is normally found in the human body or administered for dietary therapy, is an effective inhibitor of post-Amadori antigenic AGE formation, and that this inhibition is more complete than that possible by the administration of aminoguanidine.

Pyridoxine - Vitamin B_6

Vitamin B_6 is typically available in the form of pyridoxine hydrochloride in over-the-counter preparations available from many sources. For example Beach pharmaceuticals Beelith Tablets contain 25 mg of pyridoxine hydrochloride that is equivalent to 20 mg of B_6, other

preparations include Marlyn Heath Care Marlyn Formula 50 which contain 1 mg of pyridoxine HCl and Marlyn Formula 50 Mega Forte which contains 6 mg of pyridoxine HCl, Wyeth-Ayerst Stuart Prenatal® tablets which contain 2.6 mg pyridoxine HCl, J&J-Merck Corp. Stuart Formula® tablets contain 2 mg of pyridoxine HCl, and the CIBA Consumer Sunkist Children's chewable multivitamins which contain 1.05 mg of pyridoxine HCl, 150% of the U.S. RDA for children 2 to 4 years of age, and 53% of the U.S. RDA for children over 4 years of age and adults. (Physician's Desk Reference for nonprescription drugs, 14th edition (Medical Economics Data Production Co., Montvale, N.J., 1993).

There are three related forms of pyridoxine, which differ in the nature of the substitution on the carbon atom in position 4 of the pyridine nucleus: pyridoxine is a primary alcohol, pyridoxal is the corresponding aldehyde, and pyridoxamine contains an aminomethyl group at this position. Each of these three forms can be utilized by mammals after conversion by the liver into pyridoxal-5'-phosphate, the active form of the vitamin. It has long been believed that these three forms are equivalent in biological properties, and have been treated as equivalent forms of vitamin B_6 by the art. The Council on Pharmacy and Chemistry has assigned the name pyridoxine to the vitamin.

The most active antimetabolite to pyridoxine is 4-deoxypyridoxine, for which the antimetabolite activity has been attributed to the formation *in vivo* of 4-deoxypyridoxine-5-phosphate, a competitive inhibitor of several pyridoxal phosphate-dependent enzymes. The pharmacological actions of pyridoxine are limited, as it elicits no outstanding pharmacodynamic actions after either oral or intravenous administration, and it has low acute toxicity, being water soluble. It has been suggested that neurotoxicity may develop after prolonged ingestion of as little as 200 mg of pyridoxine per day. Physiologically, as a coenzyme, pyridoxine phosphate is involved in several metabolic transformations of amino acids including

decarboxylation, transamination, and racemization, as well as in enzymatic steps in the metabolism of sulfur-containing and hydroxy-amino acids. In the case of transamination, pyridoxal phosphate is aminated to pyridoxamine phosphate by the donor amino acid, and the bound pyridoxamine phosphate is then deaminated to pyridoxal phosphate by the acceptor α-keto acid. Thus vitamin B complex is known to be a necessary dietary supplement involved in specific breakdown of amino acids. For a general review of the vitamin B complex see The Pharmacological Basis of Therapeutics, 8th edition, ed. Gilman, Rall, Nies, and Taylor (Pergamon Press, New York, 1990, pp. 1293-4; pp. 1523-1540).

Surprisingly, the instant invention has discovered that effective dosages of the metabolically transitory pyridoxal amine form of vitamin B_6 (pyridoxamine), at levels above what is normally found in the human body, is an effective inhibitor of post-Amadori antigenic AGE formation, and that this inhibition may be more complete than that possible by the administration of aminoguanidine.

Formation of Stable Amadori/Schiff base Intermediary

The typical study of the reaction of a protein with glucose to form AGEs has been by ELISA using antibodies directed towards antigenic AGEs, and the detection of the production of an acid-stable fluorescent AGE, pentosidine, by HPLC following acid hydrolysis. Glycation of target proteins (i.e. BSA or RNase A) with glucose and ribose were compared by monitoring ELISA reactivity of polyclonal rabbit anti-Glucose-AGE-RNase and anti-Glucose-AGE-BSA antibodies. The antigen was generated by reacting 1 M glucose with RNase for 60 days and BSA for 90 days. The antibodies (R618 and R479) were screened and showed reactivity with only AGEs and not the protein, except for the carrier immunogen.

Example 1

Thiamine Pyrophosphate and Pyridoxamine Inhibit the Formation of Antigenic Advanced Glycation End-Products from Glucose: Comparison with Aminoguanidine

Some B_6 vitamers, especially pyridoxal phosphate (PLP), have been previously proposed to act as "competitive inhibitors" of early glycation, since as aldehydes they themselves can form Schiff bases adducts with protein amino groups (Khatami et al., 1988, *Life Sciences* **43**:1725-1731) and thus limit the amount of amines available for glucose attachment. However, effectiveness in limiting initial sugar attachment is not a predictor of inhibition of the conversion of any Amadori products formed to AGEs. The instant invention describes inhibitors of "late" glycation reactions as indicated by their effects on the *in vitro* formation of *antigenic* AGEs (Booth et al., 1996, *Biochem. Biophys. Res. Com.* **220**:113-119).

Chemicals Bovine pancreatic ribonuclease A (RNase) was chromatographically pure, aggregate-free grade from Worthington Biochemicals. Bovine Serum albumin (BSA; fraction V, fatty-acid free), human methemoglobin (Hb), D-glucose, pyridoxine, pyridoxal, pyridoxal 5'phosphate, pyridoxamine, thiamine, thiamine monophosphate, thiamine pyrophosphate, and goat alkaline phosphatase-conjugated anti-rabbit IgG were all from Sigma Chemicals. Aminoguanidine hydrochloride was purchased from Aldrich Chemicals.

Uninterrupted Glycation with Glucose Bovine serum albumin, ribonuclease A, and human hemoglobin were incubated with glucose at 37°C in 0.4 M sodium phosphate buffer of pH 7.5 containing 0.02% sodium azide. The protein, glucose (at 1.0 M), and prospective inhibitors (at 0.5, 3, 15 and 50 mM) were introduced into the incubation mixture simultaneously. Solutions were kept in the dark in capped tubes. Aliquots

were taken and immediately frozen until analyzed by ELISA at the conclusion of the reaction. The incubations were for 3 weeks (Hb) or 6 weeks (RNase, BSA).

Preparation of polyclonal antibodies to AGE proteins

Immunogen preparation followed earlier protocols (Nakayama et al., 1989, *Biochem. Biophys. Res. Comm.* **162**:740-745; Horiuchi et al., 1991, *J. Biol. Chem.* **266**:7329-7332; Makita et al., 1992, *J. Biol. Chem.* **267**:5133-5138). Briefly, immunogen was prepared by glycation of BSA (R479 antibodies) or RNase (R618 antibodies) at 1.6 g protein in 15 ml for 60-90 days using 1.5 M glucose in 0.4 M sodium phosphate buffer of pH 7.5 containing 0.05% sodium azide at pH 7.4 and 37°C. New Zealand white rabbit males of 8-12 weeks were immunized by subcutaneous administration of a 1 ml solution containing 1 mg/ml of glycated protein in Freund's adjuvant. The primary injection used the complete adjuvant and three boosters were made at three week intervals with Freund's incomplete adjuvant. Rabbits were bled three weeks after the last booster. The serum was collected by centrifugation of clotted whole blood. The antibodies are AGE-specific, being unreactive with either native proteins (except for the carrier) or with Amadori intermediates. The polyclonal anti-AGE antibodies have proven to be a sensitive and valuable analytical tool for the study of "late" AGE formation *in vitro* and *in vivo*. The nature of the dominant antigenic AGE epitope or hapten remains in doubt, although recently it has been proposed that the protein glycoxidation product carboxymethyl lysine (CmL) may be a dominant antigen of some antibodies (Reddy et al., 1995, *Biochem.* **34**:10872-10878). Earlier studies have failed to reveal ELISA reactivity with model CmL compounds (Makita et al., 1992, *J. Biol. Chem.* **267**:5133-5138).

ELISA detection of AGE products The general method of Engvall (1981, *Methods Enzymol.* **70**:419-439) was used to

perform the ELISA. Typically, glycated protein samples were diluted to approximately 1.5 ug/ml in 0.1 M sodium carbonate buffer of pH 9.5 to 9.7. The protein was coated overnight at room temperature onto 96-well polystyrene plates by pippetting 200 ul of the protein solution in each well (0.3 ug/well). After coating, the protein was washed from the wells with a saline solution containing 0.05% Tween-20. The wells were then blocked with 200 ul of 1% casein in carbonate buffer for 2 h at 37°C followed by washing. Rabbit anti-AGE antibodies were diluted at a titer of about 1:350 in incubation buffer, and incubated for 1 h at 37°C, followed by washing. In order to minimize background readings, antibodies R479 used to detect glycated RNase were raised against glycated BSA, and antibodies R618 used to detect glycated BSA and glycated Hb were raised against glycated RNase. An alkaline phosphatase-conjugated antibody to rabbit IgG was then added as the secondary antibody at a titer of 1:2000 or 1:2500 (depending on lot) and incubated for 1 h at 37°C, followed by washing. The p-nitrophenylphosphate substrate solution was then added (200 ul/well) to the plates, with the absorbance of the released p-nitrophenolate being monitored at 410 nm with a Dynatech MR 4000 microplate reader.

Controls containing unmodified protein were routinely included, and their readings were subtracted, the corrections usually being negligible. The validity of the use of the ELISA method in quantitatively studying the kinetics of AGE formation depends on the linearity of the assay (Kemeny & Challacombe, 1988, *ELISA and Other Solid Phase Immunoassays*, John Wiley & Sons, Chichester, U.K.). Control experiments were carried out, for example, demonstrating that the linear range for RNase is below a coating concentration of about 0.2-0.3 mg/well.

Results

Figure 1 A-D are graphs which show the effect of vitamin B_6 derivatives on post-Amadori AGE formation in

bovine serum albumin glycated with glucose. BSA (10 mg/ml) was incubated with 1.0 M glucose in the presence and absence of the various indicated derivative in 0.4 M sodium phosphate buffer of pH 7.5 at 37°C for 6 weeks. Aliquots were assayed by ELISA using R618 anti-AGE antibodies. Concentrations of the inhibitors were 3, 15 and 50 mM. Inhibitors used in Figures (1A) Pyridoxamine (PM); (1B) pyridoxal phosphate (PLP); (1C) pyridoxal (PL); (1D) pyridoxine (PN).

Figure 1 (control curves) demonstrates that reaction of BSA with 1.0 M glucose is slow and incomplete after 40 days, even at the high sugar concentration used to accelerate the reaction. The simultaneous inclusion of different concentrations of various B_6 vitamers markedly affects the formation of post-Amadori antigenic AGEs. (Figure 1A-D) Pyridoxamine and pyridoxal phosphate strongly suppressed post-Amadori antigenic AGE formation at even the lowest concentrations tested, while pyridoxal was effective above 15 mM. Pyridoxine was slightly effective at the highest concentrations tested.

Figure 2 A-D are graphs which show the effect of vitamin B_1 derivatives and aminoguanidine (AG) on post-Amadori AGE formation in bovine serum albumin. BSA (10 mg/ml) was incubated with 1.0 M glucose in the presence and absence of the various indicated derivative in 0.4 M sodium phosphate buffer of pH 7.5 at 37°C for 6 weeks. Aliquots were assayed by ELISA using R618 anti-AGE antibodies. Concentrations of the inhibitors were 3, 15 and 50 mM. Inhibitors used in Figures (2A) Thiamine pyrophosphate (TPP); (2B) thiamine monophosphate (TP); (2C) thiamine (T); (2D) aminoguanidine (AG).

Of the various B_1 vitamers similarly tested (Figure 2A-D), thiamine pyrophosphate was effective at all concentrations tested (Figure 2C), whereas thiamine and thiamine monophosphate were not inhibitory. Most significantly it is remarkable to note the decrease in the *final levels* of AGEs formed observed with thiamine

pyrophosphate, pyridoxal phosphate and pyridoxamine. Aminoguanidine (Figure 2D) produced some inhibition of post-Amadori AGE formation in BSA, but less so than the above compounds. Similar studies were carried out with human methemaglobin and bovine ribonuclease A.

Figure 3 A-D are graphs which show the effect of vitamin B_6 derivatives on post-Amadori AGE formation in human methemoglobin. Hb (1 mg/ml) was incubated with 1.0 M glucose in the presence and absence of the various indicated derivative in 0.4 M sodium phosphate buffer of pH 7.5 at 37°C for 3 weeks. Aliquots were assayed by ELISA using R618 anti-AGE antibodies. Concentrations of the inhibitors were 0.5, 3, 15 and 50 mM. Inhibitors used in Figures (3A) Pyridoxamine (PM); (3B) pyridoxal phosphate (PLP); (3C) pyridoxal (PL); (3D) pyridoxine (PN).

It had been previously reported that Hb of a diabetic patient contains a component that binds to anti-AGE antibodies, and it was proposed that this glycated Hb (termed Hb-AGE, not to be confused with Hb_{A1c}) could be useful in measuring long-term exposure to glucose. The *in vitro* incubation of Hb with glucose produces antigenic AGEs at an apparently faster rate than observed with BSA. Nevertheless, the different B_6 (Figure 3A-D) and B_1 (Figure 4A-C) vitamers exhibited the same inhibition trends in Hb, with pyridoxamine and thiamine pyrophosphate being the most effective inhibitors in each of their respective families. Significantly, in Hb, aminoguanidine only inhibited the rate of post-Amadori AGE formation, and not the final levels of post-Amadori AGE formed (Figure 4D).

With RNase the rate of post-Amadori antigenic AGE formation by glucose was intermediate between that of Hb and BSA, but the extent of inhibition within each vitamer series was maintained. Again pyridoxamine and thiamine pyrophosphate were more effective that aminoguanidine (Figure 5).

Figure 4 A-D are graphs which show the effect of vitamin B_1 derivatives and aminoguanidine (AG) on post-

Amadori AGE formation in human methemoglobin. Hb (1 mg/ml) was incubated with 1.0 M glucose in the presence and absence of the various indicated derivative in 0.4 M sodium phosphate buffer of pH 7.5 at 37°C for 3 weeks. Aliquots were assayed by ELISA using R618 anti-AGE antibodies. Concentrations of the inhibitors were 0.5, 3, 15 and 50 mM. Inhibitors used in Figures (4A) Thiamine pyrophosphate (TPP); (4B) thiamine monophosphate (TP); (4C) thiamine (T); (4D) aminoguanidine (AG).

Figure 5 is a bar graph which shows a comparison of the inhibition of the glycation of ribonuclease A by thiamine pyrophosphate (TPP), pyridoxamine (PM) and aminoguanidine (AG). RNase (1 mg/ml) was incubated with 1.0 M glucose (glc) in the presence and absence of the various indicated derivative in 0.4 M sodium phosphate buffer of pH 7.5 at 37°C for 6 weeks. Aliquots were assayed by ELISA using R479 anti-AGE antibodies. The indicated percent inhibition was computed from ELISA readings in the absence and presence of the inhibitors at the 6 week time point. Concentrations of the inhibitors were 0.5, 3, 15 and 50 mM.

Discussion

These results demonstrate that certain derivatives of B_1 and B_6 vitamins are capable of inhibiting "late" post-Amadori AGE formation. Some of these vitamers successfully inhibited the final levels of post-Amadori AGE produced, in contrast to aminoguanidine, suggesting that they have greater interactions with Amadori or post-Amadori precursors to antigenic AGEs. The Amadori and post-Amadori intermediates represent a crucial juncture where the "classical" pathway of nonenzymatic glycation begins to become essentially irreversible (Scheme I). In earlier inhibition studies "glycation" was usually measured either as Schiff base formed (after reduction with labeled cyanoborohydride) or as Amadori product formed (after acid precipitation using labeled sugar). Such assays do not yield information on inhibition of post-Amadori conversion steps

to "late" post-Amadori AGE products, since such steps lead to no change in the amount of labeled sugar that is attached to the proteins. Other "glycation" assays have relied on the sugar-induced increase of non-specific protein fluorescence, but this can also be induced by dicarbonyl oxidative fragments of free sugar, such as glycoaldehyde or glyoxal (Hunt et al., 1988, *Biochem.* **256**:205-212), independently of Amadori product formation.

In the case of pyridoxal (PL) and pyridoxal phosphate (PLP), the data support the simple mechanism of inhibition involving competitive Schiff-base condensation of these aldehydes with protein amino groups at glycation sites. Due to internal hemiacetal formation in pyridoxal but not pyridoxal phosphate, stronger inhibition of post-Amadori AGE formation by PLP is expected by this competitive mechanism. This indeed is observed in the data (Figure 1B, 1C, Figure 3B, 3C). The inhibition by pyridoxamine is necessarily different, as pyridoxamine lacks an aldehyde group. However, pyridoxamine is a candidate amine potentially capable of forming a Schiff-base linkage with the carbonyls of open-chain sugars, with dicarbonyl fragments, with Amadori products, or with post-Amadori intermediates. The mechanism of inhibition of B_1 compounds is not obvious. All the forms contain an amino functionality, so that the marked efficiency of only the pyrophosphate form suggests an important requirement for a strong negative charge.

A significant unexpected observation is that the extent of inhibition by aminoguanidine, and some of the other compounds, is considerably less at late stages of the reaction, than during the early initial phase. This suggests a different mechanism of action than that of pyridoxamine and thiamine pyrophosphate, suggesting that the therapeutic potential of these compounds will be enhanced by co-administration with aminoguanidine.

Example 2

Kinetics of Non-enzymatic glycation: Paradoxical Inhibition by Ribose and Facile Isolation of Protein Intermediate for Rapid Post-Amadori AGE Formation

While high concentrations of glucose are used to cause the non-enzymatic glycation of proteins, paradoxically, it was found that ribose at high concentrations is *inhibitory* to post-Amadori AGE formation in ribonuclease by acting on the post-Amadori "late" stages of the glycation reaction. This unexpectedly inhibitory effect suggests that the "early" reactive intermediates, presumably Amadori products, can be accumulated with little formation of "late" post-Amadori AGE products (AGEs; Maillard products). Investigation into this phenomenon has demonstrated: (1) ability to define conditions for the kinetic isolation of Amadori (or post-Amadori) glycated intermediate(s); (2) the ability study the fast kinetics of buildup of such an intermediate; (3) the ability to study the surprisingly rapid kinetics of conversion of such intermediates to AGE products *in the absence of free or reversibly bound sugar;* (4) the ability to use these intermediates to study and characterize inhibition of post-Amadori steps of AGE formation thus providing a novel system to investigate the mechanism of reaction and the efficacy of potential agents that could block AGE formation; and (5) with this knowledge it is also further possible to use ^{13}C NMR to study the reactive intermediates and monitor their conversion to various candidate AGEs (Khalifah et al., 1996, *Biochemistry* **35(15)**:4645-4654).

Chemicals and Materials As in Example 1 above.

Preparation of polyclonal antibodies to AGEs

As in Example 1 above.

ELISA detection of AGE products As in Example 1 above.

Amino Acid Analysis Amino acid analyses were carried out at the Biotechnology Support Facility of the Kansas University Medical Center. Analyses were performed after hydrolysis of glycated protein (reduced with sodium cyanoborohydride) with 6 N HCl at 110°C for 18-24 h. Phenyl isothiocyanate was used for derivatization, and PTH derivatives were analyzed by reverse-phase HPLC on an Applied Biosystems amino acid analyzer (420A derivatizer, 130A separation system, 920A data analysis system).

Pentosidine Reverse-Phase HPLC Analysis Pentosidine production in RNase was quantitated by HPLC (Sell & Monnier, 1989, *J. Biol. Chem.* **264**:21597-21602; Odetti et al., 1992, *Diabetes* **41**:153-159). Ribose-modified protein samples were hydrolyzed in 6 N HCl for 18 h at 100°C and then dried in a Speed Vac. The samples were then redissolved, and aliquots were taken into 0.1% trifluoroacetic acid and analyzed by HPLC on a Shimadzu system using a Vydac C-18 column equilibrated with 0.1% TFA. A gradient of 0-6% acetonitrile (0.1% in TFA) was run in 30 min at a flow rate of about 1 ml/min. Pentosidine was detected by 335 nm excitation/385 nm emission fluorescence, and its elution time was determined by running a synthesized standard. Due to the extremely small levels of pentosidine expected (Grandhee & Monnier, 1991, *J. Biol. Chem.* **266**:11649-11653; Dyer et al., 1991, *J. Biol. Chem.* **266**:11654-11660), no attempt was made to quantitate the absolute concentrations. Only relative concentrations were determined from peak areas.

Glycation Modifications Modification with ribose or glucose was generally done at 37°C in 0.4 M phosphate buffer of pH 7.5 containing 0.02% sodium azide. The high buffer concentration was always used with 0.5 M ribose modifications. The solutions were kept in capped tubes and opened only to remove timed aliquots that were immediately frozen for later carrying out the various analyses. "Interrupted glycation" experiments were carried out by

first incubating protein with the ribose at 37°C for 8 or 24 h, followed by immediate and extensive dialysis against frequent cold buffer changes at 4°C. The samples were then reincubated by quickly warming to 37°C in the absence of external ribose. Aliquots were taken and frozen at various intervals for later analysis. Due to the low molecular weight of RNase, protein concentrations were remeasured after dialysis even when low molecular weight cut-off dialysis tubing was used. An alternative procedure was also devised (see below) in which interruption was achieved by simple 100-fold dilution from reaction mixtures containing 0.5 M ribose. Protein concentrations were estimated from UV spectra. The difference in molar extinction between the peak (278 nm) and trough (250 nm) was used for RNase concentration determinations in order to compensate for the general increase in UV absorbance that accompanies glycation. Time-dependent UV-difference spectral studies were carried out to characterize the glycation contributions of the UV spectrum.

Data Analysis and Numerical Simulations of Kinetics

Kinetic data were routinely fit to monoexponential or biexponential functions using nonlinear least-squares methods. The kinetic mechanisms of Schemes 5-6 have been examined by numerical simulations of the differential equations of the reaction. Both simulations and fitting to observed kinetics data were carried out with the SCIENTIST 2.0 software package (Micromath, Inc.). Determination of apparent half-times (Figure 6B) from kinetic data fit to two-exponential functions (Figure 6A) was carried out with the "solve" function of MathCAD 4.0 software (MathSoft, Inc.).

RESULTS

Comparison of Glycation by Glucose and Ribose

The reaction of RNase A with ribose and glucose has been followed primarily with ELISA assays, using R479 rabbit

AGE-specific antibodies developed against glucose-modified BSA. To a lesser extent, the production of pentosidine, the only known acid-stable fluorescent AGE, was quantiated by HPLC following acid hydrolysis. Preliminary studies using 0.05 M ribose at 37°C showed that the rate of antigenic AGE formation appears to be modestly increased (roughly 2-3 fold as measured by the apparent half-time) as the pH is increased from 5.0 to 7.5, with an apparent small induction period at the beginning of the kinetics in all cases. The glycation of RNase with 0.05 M ribose at pH 7.5 (half-time near 6.5 days) appears to be almost an order of magnitude faster than that of glycation with 1.0 M glucose (half-time in excess of 30 days; see Figure 7B, solid line). The latter kinetics also displayed a small induction period but incomplete leveling off even after 60 days, making it difficult to estimate a true half-time.

When the dependence of the kinetics on ribose concentration was examined at pH 7.5, a most unexpected result was obtained. The rate of reaction initially increased with increasing ribose concentration, but at concentrations above 0.15 M the rate of reaction leveled off and then significantly decreased (Figure 6A). A plot of the dependence of the reciprocal half-time on the concentration of ribose (Figure 6B) shows that high ribose concentrations are paradoxically inhibitory to post-Amadori antigenic AGE formation. This unusual but consistent effect was found to be independent of changes in the concentration of either buffer (2-fold) or RNase (10-fold), and it was not changed by affinity purification of the R479 antibody on a column of immobilized AGE-RNase. It is also not due to effects of ribose on the ELISA assay itself. The measured inhibitory effect by ribose on post-Amadori AGE formation is not likely due to ribose interference with antibody recognition of the AGE antigenic sites on protein in the ELISA assay. Prior to the first contact with the primary anti-AGE antibody on the ELISA plates, glycated protein has been diluted over 1000-fold, washed extensively with Tween-20 after adsorption, and

blocked with a 1% casein coating followed by further washing with Tween-20.

Kinetics of Formation of post-Amadori Antigenic AGEs by "Interrupted Glycation"

In view of the small induction period seen, an attempt was made to determine whether there was some accumulation during the reaction, of an early precursor such as an Amadori intermediate, capable of producing the ELISA-detectable post-Amadori antigenic AGEs. RNase was glycated at pH 7.5 and 37°C with a high ribose concentration of 0.5 M, and the reaction was interrupted after 24 h by immediate cooling to 4°C and dialysis against several changes of cold buffer over a period of 24 h to remove free and reversibly bound (Schiff base) ribose. Such a ribose-free sample was then rapidly warmed to 37°C *without re-adding any ribose*, and was sampled for post-Amadori AGE formation over several days. The AGE antigen production of this 24 h "interrupted glycation" sample is shown by the dashed line and open triangles in Figure 7A, the time spent in the cold dialysis is not included. An uninterrupted control (solid line and filled circles) is also shown for comparison. Dramatically different kinetics of post-Amadori antigenic AGE formation are evident in the two samples. The kinetics of AGE antigen production of the ribose-free interrupted sample now show (1) monoexponential kinetics with no induction period, (2) a greatly enhanced rate of antigenic AGE formation, with remarkable half-times of the order of 10 h, and (3) production of levels of antigen comparable to those seen in long incubations in the continued presence of ribose (see Figure 6A). Equally significant, the data also demonstrate that negligible AGE antigen was formed during the cold dialysis period, as shown by the small difference between the open triangle and filled circle points at time 1 day in Figure 7A. Very little, if any, AGE was formed by the "interruption" procedure itself. These observations show that a fully competent isolatable intermediate or precursor

to antigenic AGE has been generated during the 24 h contact with ribose prior to the removal of the free and reversibly bound sugar.

Samples interrupted after only 8 h produced a final amount of AGE antigen that was about 72% of the 24 h interrupted sample. Samples of RNase glycated with only 0.05 M ribose and interrupted at 8 h by cold dialysis and reincubation at 37°C revealed less than 5% production of ELISA-reactive antigen after 9 days. Interruption at 24 h, however, produced a rapid rise of ELISA antigen (similar to Figure 7A) to a level roughly 50% of that produced in the uninterrupted presence of 0.05 M ribose.

The same general interruption effects were also seen with other proteins (BSA and Hemoglobin). Except for a somewhat different absolute value of the rate constants, and the amount of antigenic AGEs formed during the 24 h 0.5 M ribose incubation, the same dramatic increase in the rate of AGE antigen formation was observed after removal of 0.5 M ribose.

Glycation is much slower with glucose than with ribose (note the difference in time scales between Figure 7A and Figure 7B). However, unlike the case with ribose, interruption after 3 days of glycation by 1.0 M glucose produced negligible buildup of precursor to ELISA-reactive AGE antigens (Figure 7B, dashed curve).

Kinetics of Pentosidine Formation

The samples subjected to ELISA testing were also assayed for the production of pentosidine, an acid-stable AGE. The content of pentosidine was measured for the same RNase samples analyzed for antibody reactivity by ELISA. Glycation by ribose in 0.4 M phosphate buffer at pH 7.5 produced pentosidine in RNase A that was quantitated by fluroescence after acid hydrolysis. Figure 8A shows that under uninterrupted conditions, 0.05 M ribose produces a progressive increase in pentosidine. However, when glycation is carried out under "interrupted" conditions

using 0.5 M ribose, a dramatic increase in the rate of pentosidine formation is seen immediately after removal of excess ribose (Figure 8B), which is similar to, but slightly more rapid than, the kinetics of the appearance of antigenic AGEs (Figure 7A). A greater amount of pentosidine was also produced with 24 h interruption as compared with 8 h. Reproducible differences between the kinetics of formation of pentosidine and antigenic AGEs can also be noted. A significant amount of pentosidine is formed during the 24 h incubation and also during the cold dialysis, resulting in a jump of the dashed vertical line in Figure 8B. Our observations thus demonstrate that a pentosidine precursor accumulates during ribose glycation that can rapidly produce pentosidine after ribose removal (cf. Odetti et al., 1992, *Diabetes* **41**:153-159).

Rate of Buildup of the Reactive Intermediate(s)

The "interrupted glycation" experiments described above demonstrate that a precursor or precursors to both post-Amadori antigenic AGEs and pentosidine can be accumulated during glycation with ribose. The kinetics of formation of this intermediate can be independently followed and quantitated by a variation of the experiments described above. The amount of intermediate generated in RNase at different contact times with ribose can be assayed by the maximal extent to which it can produce antigenic AGE after interruption. At variable times after initiating glycation, the free and reversibly-bound ribose is removed by dialysis in the cold or by rapid dilution (see below). Sufficient time (5 days, which represents several half-lives according to Figure 7A) is then allowed after warming to 37°C for maximal development of post-Amadori antigenic AGEs. The ELISA readings 5 days after each interruption point, representing maximal AGE development, would then be proportional to the intermediate concentration present at the time of interruption.

Figure 9 shows such an experiment where the kinetics of

intermediate buildup are measured for RNase A in the presence of 0.5 M ribose (solid symbols and curve). For comparison, the amount of AGE present before ribose removal at each interruption point is also shown (open symbols and dashed lines). As expected (cf. Figure 7A), little AGE is formed prior to removal (or dilution) of ribose, so that ELISA readings after the 5 day secondary incubation periods are mostly a measure of AGE formed after ribose removal. The results in Figure 9 show that the rate of buildup of intermediate in 0.5 M ribose is exponential and very fast, with a half-time of about 3.3 h. This is about 3-fold more rapid than the observed rate of conversion of the intermediate to antigenic AGEs after interruption (open symbols and dashed curve Figure 7A).

In these experiments the removal of ribose at each interruption time was achieved by 100-fold dilution, and not by dialysis. Simple dilution reduced the concentration of ribose from 0.05 M to 0.005 M. It was independently determined (Figure 6A) that little AGE is produced in this time scale with the residual 5 mM ribose. This dilution approach was primarily dictated by the need for quantitative point-to-point accuracy. Such accuracy would not have been achieved by the dialysis procedure that would be carried out independently for each sample at each interruption point. Our results show that dilution was equivalent to dialysis.

A separate control experiment (see Figure 10 below) demonstrated that the instantaneous 100-fold dilution gave nearly identical results to the dialysis procedure. These control experiments demonstrate that the reversible ribose-protein binding (Schiff base) equilibrium is quite rapid on this time scale. This is consistent with data of Bunn and Higgins (1981, *Science* **213**: 222-224) that indicated that the half-time of Schiff base formation with 0.5 M ribose should be on the order of a few minutes. The 100-fold rapid dilution method to reduce ribose is a valid method where quantitative accuracy is essential and cannot be achieved by multiple dialysis of many samples.

Direct Inhibition of Post-Amadori AGE Formation from the Intermediate by Ribose and Glucose

The increase in the rate of AGE formation after interruption and sugar dilution suggests, but does not prove, that high concentrations of ribose are inhibiting the reaction at or beyond the first "stable" intermediate, presumably the Amadori derivative (boxed in Scheme I). A test of this was then carried out by studying the effect of directly adding ribose, on the post-Amadori reaction. RNase was first incubated for 24 h in 0.5 M ribose in order to prepare the intermediate. Two protocols were then carried out to measure possible inhibition of the post-Amadori formation of antigenic AGEs by different concentrations of ribose. In the first experiment, the 24 h ribated sample was simply diluted 100-fold into solutions containing varying final concentrations of ribose ranging from 0.005 M to 0.505 M (Figure 10A). The rate and extent of AGE formation are clearly seen to be diminished by increasing ribose concentrations. Significantly, up to the highest concentration of 0.5 M ribose, the kinetics appear exponential and do not show the induction period that occurs with uninterrupted glycation (Figures 6A and 7A) in high ribose concentrations.

A second experiment (Figure 10B) was also conducted in which the 24 h interrupted sample was extensively dialyzed in the cold to release free and reversibly bound ribose as well as any inhibitory products that may have formed during the 24 h incubation with ribose. Following this, aliquots were diluted 100-fold into varying concentrations of freshly made ribose, and the formation of antigenic AGE products was monitored as above. There results were nearly identical to the experiment of Figure 10A where the dialysis step was omitted. In both cases, the rate and extent of AGE formation were diminished by increasing concentrations of ribose, and the kinetics appeared exponential with no induction period.

The question of whether glucose or other sugars can

also inhibit the formation of AGEs from the reactive intermediate obtained by interrupted glycation in 0.5 M ribose was also investigated. The effects of glucose at concentrations of 1.0-2.0 M were tested (data not shown). Glucose was clearly not as inhibitory as ribose. When the 24 h ribose interrupted sample was diluted 100-fold into these glucose solutions, the amount of antigenic AGE formed was diminished by about 30%, but there was little decrease in the apparent rate constant. Again, the kinetics appeared exponential.

Effect of pH on Post-Amadori Kinetics of AGE Formation

The interrupted glycation method was used to investigate the pH dependence of the post-Amadori kinetics of AGE formation from the reactive intermediate. In these experiments, RNase A was first reacted for 24 h with 0.5 M ribose at pH 7.5 to generate the reactive intermediate. The kinetics of the decay of the intermediate to AGEs were then measured by ELISA. Figure 11 shows that an extremely wide pH range of 5.0-9.5 was achievable when the kinetics were measured by initial rates. A remarkable bell-shaped dependence was observed, showing that the kinetics of antigenic AGEs formation are decreased at both acidic and alkaline pH ranges, with an optimum near pH 8.

A single "pH jump" experiment was also carried out on the pH 5.0 sample studied above which had the slowest rate of antigenic AGE formation. After 12 days at 37°C in pH 5.0 buffer, the pH was adjusted quickly to 7.5, and antigenic AGE formation was monitored by ELISA. Within experimental error, the sample showed identical kinetics (same first order rate constant) of AGE formation to interrupted glycation samples that had been studied directly at pH 7.5 (Figure 12). In this experiment, the relative amounts of antigenic AGE could not be directly compared on the same ELISA plate, but the pH-jumped sample appeared to have formed substantial though somehow diminished levels of antigenic AGEs. These results demonstrate that intermediate

can be prepared free of AGE and stored at pH 5 for later studies of conversion to AGEs.

Inhibition of Post-Amadori AGE formation by Aminoguanidine

The efficacy of aminoguanidine was tested by this interrupted glycation method, i.e., by testing its effect on post-Amadori formation of antigenic AGEs after removal of excess and reversibly bound ribose. Figure 20A demonstrates that aminoguanidine has modest effects on blocking the formation of antigenic AGEs in RNase under these conditions, with little inhibition below 50 mM. Approximately 50% inhibition is achieved only at or above 100-250 mM. Note again that in these experiments, the protein was exposed to aminoguanidine only after interruption and removal of free and reversibly bound ribose. Comparable results were also obtained with the interrupted glycation of BSA (Figure 20B).

Amino acid analysis of Interrupted Glycation Samples

Amino acid analysis was carried out on RNase after 24 h contact with 0.5 M ribose (undialyzed), after extensive dialysis of the 24 h glycated sample, and after 5 days of incubation of the latter sample at 37°C. These determinations were made after sodium cyanoborohydride reduction, which reduces Schiff base present on lysines or the terminal amino group. All three samples, normalized to alanine (12 residues), showed the same residual lysine content (4.0 ± 0.5 out of the original 10 in RNase). This indicates that after 24 h contact with 0.5 M ribose, most of the formed Schiff base adducts had been converted to Amadori or subsequent products. No arginine or histidine residues were lost by modification.

Discussion

The use of rapidly reacting ribose and the discovery of its reversible inhibition of post-Amadori steps have permitted the dissection and determination of the kinetics of different steps of protein glycation in RNase. Most

previous kinetic studies of protein "glycation" have actually been restricted to the "early" steps of Schiff base formation and subsequent Amadori rearrangement. Some kinetic studies have been carried out starting with synthesized fructosylamines, i.e. small model Amadori compounds of glucose (Smith and Thornalley, 1992, *Eur. J. Biochem.* **210**:729-739, and references cited therein), but such studies, with few exceptions, have hitherto not been possible with proteins. One notable exception is the demonstration by Monnier (Odetti et al., 1992, *supra*) that BSA partially glycated with ribose can rapidly produce pentosidine after ribose removal. The greater reactivity of ribose has also proven a distinct advantage in quantitatively defining the time course of AGE formation. It is noted that glucose and ribose are both capable of producing similar AGE products, such as pentosidine (Grandhee & Monnier, 1991, *supra;* Dyer et al. 1991, *supra*), and some ^{13}C NMR model compound work has been done with ADP-ribose (Cervantes-Laurean et al., 1993, *Biochemistry* **32**:1528-1534). The present work shows that antigenic AGE products of ribose fully cross-react with anti-AGE antibodies directed against glucose-modified proteins, suggesting that ribose and glucose produce similar antigenic AGEs. The primary kinetic differences observed between these two sugars are probably due to relative differences in the rate constants of steps leading to post-Amadori AGE formation, rather than in the mechanism.

The results presented reveal a marked and paradoxical inhibition of overall AGE formation by high concentrations of ribose (Figure 6) that has not been anticipated by earlier studies. This inhibition is rapidly reversible in the sense that it is removed by dialysis of initially modified protein (Figure 7A) or by simple 100-fold dilution (as used in Figure 11). The experiments of Figure 10 demonstrate that it is not due to the accumulation of dialyzable inhibitory intermediates during the initial glycation, since dialysis of 24 h modified protein followed

by addition of different concentrations of fresh ribose induces the same inhibition. The data of Figure 10A,B show that the inhibition occurs by reversible and rapid interaction of ribose with protein intermediate containing reactive Amadori products. The inhibition is unlikely to apply to the early step of formation of Amadori product due to the rapid rate of formation of the presumed Amadori intermediate that was determined in the experiment of Figure 9. The identification of the reactive intermediate as an Amadori product is well supported by the amino acid analysis carried out (after sodium cyanoborohydrate reduction) before and after dialysis at the 24 h interruption point. The unchanged residual lysine content indicates that any dischageable Schiff bases have already been irreversibly converted (presumably by Amadori rearrangement) by the 24 h time.

The secondary ribose suppression of "late" but not "early" glycation steps significantly enhances the accumulation of a fully-competent reactive Amadori intermediate containing little AGE. Its isolation by the interruption procedure is of importance for kinetic and structural studies, since it allows one to make studies in the absence of free or Schiff base bound sugar and their attendant reactions and complications. For example, the post-Amadori conversion rates to antigenic AGE and pentosidine AGE products have been measured (Figure 7A, open symbols, and Figure 8B), and demonstrated to be much faster (t 1/2 ~ 10 h) than reflected in the overall kinetics under uninterrupted conditions (Figure 6A and Figure 8A). The rapid formation of pentosidine that was measured appears consistent with an earlier interrupted ribose experiment on BSA by Odetti et al. (1992, *supra*). Since ribose and derivatives such as ADP-ribose are normal metabolites, the very high rates of AGE formation seen here suggest that they should be considered more seriously as sources of potential glycation in various cellular compartments (Cervantes-Laurean et al., 1993, *supra*), even though their

concentrations are well below those of the less reactive glucose.

Another new application of the isolation of intermediate is in studying the pH dependence of this complex reaction. The unusual bell-shaped pH profile seen for the post-Amadori AGE formation (Figure 11) is in striking contrast to the mild pH dependence of the overall reaction. The latter kinetics reflect a composite effect of pH on all steps in the reaction, including Schiff base and Amadori product formation, each of which may have a unique pH dependence. This complexity is especially well illustrated by studies of hemoglobin glycation (Lowery et al., 1985, *J. Biol. Chem.* **260**:11611-11618). The bell-shaped pH profile suggests, but does not prove, the involvement of two ionizing groups. If true, the data may imply the participation of a second amino group, such as from a neighboring lysine, in the formation of dominant antigenic AGEs. The observed pH profile and the pH-jump observations described suggest that a useful route to isolating and maintaining the reactive intermediate would be by the rapid lowering of the pH to near 5.0 after 24 h interruption.

The kinetic studies provide new insights into the mechanisms of action of aminoguanidine (guanylhydrazine), an AGE inhibitor proposed by Cerami and co-workers to combine with Amadori intermediates (Brownlee et al., 1986, *supra*). This proposed pharmacological agent is now in Phase III clinical trials for possible therapeutic effects in treating diabetes (Vlassara et al., 1994, *supra*). However, its mechanism of AGE inhibition is likely to be quite complex, since it is multifunctional. As a nucelophilic hydrazine, it can reversibly add to active carbonyls, including aldehydo carbonyls of open-chain glucose and ribose (Khatami et al., 1988, *Life Sci.* **43**:1725-1731; Hirsch et al., 1995, *Carbohyd. Res.* **267**:17-25), as well as keto carbonyls of Amadori compounds. It is also a guanidinium compound that can scavange highly reactive dicarbonyl glycation intermediates such as glyoxal and glucosones (Chen & Cerami, 1993, *J.*

Carbohyd. Chem. **12**:731-742; Hirsch et al., 1992, *Carbohyd. Res.* **232**:125-130; Ou & Wolff, 1993, *Biochem. Pharmacol.* **46**:1139-1144). The interrupted glycation method allowed examination of aminoguanidine efficacy on only post-Amadori steps of AGE formation. Equally important, it allowed studies in the absence of free sugar or dicarbonyl-reactive fragments from free sugar (Wolff & Dean, 1987, *Biochem. J.* **245**:243-250; Wells-Knecht et al., 1995, *Biochemistry* **34**:3702-3709) that can combine with aminoguanidine. The results of Figure 20 demonstrate that aminoguanidine has, at best, only a modest effect on post-Amadori AGE formation reactions, achieving 50% inhibition at concentrations above 100-250 mM. The efficacy of aminoguanidine thus predominantly arises either from inhibiting early steps of glycation (Schiff base formation) or from scavenging highly reactive dicarbonyls generated during glycation. Contrary to the original claims, it does not appear to inhibit AGE formation by complexing the Amadori intermediate.

The use of interrupted glycation is not limited for kinetic studies. Interrupted glycation can simplify structural studies of glycated proteins and identifying unknown AGEs using techniques such as ^{13}C NMR that has been used to detect Amadori adducts of RNase (Neglia et al., 1983, *J. Biol. Chem.* **258**:14279-14283; 1985, *J. Biol. Chem.* **260**:5406-5410). The combined use of structural and kinetic approaches should also be of special interest. For example, although the identity of the dominant antigenic AGEs reacting with the polyclonal antibodies remains uncertain, candidate AGEs, such as the recently proposed (carboxymethyl)lysine (Reddy et al., 1995, *Biochemistry* **34**:10872-10878; cf. Makita et al., 1992, *J. Biol. Chem.* **267**:5133-5138) should display the same kinetics of formation from the reactive intermediate that we have observed. The availability of the interrupted kinetics approach will also help to determine the importance of the Amadori pathway to the formation of this AGE. Similarly, monitoring of the interrupted glycation reaction by

techniques such as ^{13}C NMR should identify resonances of other candidate antigenic AGEs as being those displaying similar kinetics of appearance. Table I lists the ^{13}C NMR peaks of the Amadori intermediate of RNase prepared by reaction with C-2 enriched ribose. The downfield peak near 205 ppm is probably due to the carbonyl of the Amadori product. In all cases, the ability to remove excess free and Schiff base sugars through interrupted glycation will considerably simplify isolation, identification, and structural characterization.

Table I lists the peaks that were assigned to the Post-Amadori Intermediate due to their invariant or decreasing intensity with time. Peak positions are listed in ppm downfield from TMS.

Table I 125MHz C-13 NMR Resonances of Ribonuclease Amadori Intermediate Prepared by 24 HR Reaction with 99% [2-C13]Ribose

216.5	ppm	108.5	ppm
211.7		105.9	
208		103.9	
		103	
172		95.8	
165			
163.9		73.65	
162.1		70.2	
		69.7	

Ribonuclease A was reacted for 24 hr with 0.5 M ribose 99% enriched at C-2, following which excess and Schiff base bound ribose was removed by extensive dialysis in the cold. The sample was then warmed back to 37°C immediately before taking a 2 hr NMR scan. The signals from RNase reacted with natural abundance ribose under identical conditions were then subtracted from the NMR spectrum. Thus all peaks shown are due to enriched C-13 that originated at the C-2 position. Some of the peaks arise from degradation products

of the intermediate, and these can be identified by the increase in the peak intensity over time. Figure 31 shows the NMR spectrum obtained.

Example 3

***In Vitro* Inhibition of the Formation of Antigenic Advanced Glycation End-Products (AGEs) by Derivatives of Vitamins B_1 and B_6 and Aminoguanidine. Inhibition of Post-Amadori Kinetics Differs from that of Overall Glycation**

The interrupted glycation method for following post-Amadori kinetics of AGE formation allows for the rapid quantitative study of "late" stages of the glycation reaction. Importantly, this method allows for inhibition studies that are free of pathways of AGE formation which arise from glycoxidative products of free sugar or Schiff base (Namiki pathway) as illustrated in Scheme I. Thus the interrupted glycation method allows for the rapid and unique identification and characterization of inhibitors of "late" stages of glycation which lead to antigenic AGE formation.

Among the vitamin B_1 and B_6 derivatives examined, pyridoxamine and thiamine pyrophosphate are unique inhibitors of the post-Amadori pathway of AGE formation. Importantly, it was found that efficacy of inhibition of overall glycation of protein, in the presence of high concentrations of sugar, is not predictive of the ability to inhibit the post-Amadori steps of AGE formation where free sugar is removed. Thus while pyridoxamine, thiamine pyrophosphate and aminoguanidine are potent inhibitors of AGE formation in the overall glycation of protein by glucose, aminoguanidine differs from the other two in that it is not an effective inhibitor of post-Amadori AGE formation. Aminoguanidine markedly slows the initial rate of AGE formation by ribose under uninterrupted conditions, but has no effect on the final levels of antigenic AGEs produced. Examination of different proteins (RNase, BSA and hemoglobin), confirmed that the inhibition results are

generally non-specific as to the protein used, even though there are individual variations in the rates of AGE formation and inhibition.

Chemicals and Materials As in Example 1 above.

Preparation of polyclonal antibodies to AGEs
As in Example 1 above.

ELISA detection of AGE products As in Example 1 above.

Uninterrupted ribose glycation assays Bovine serum albumin, ribonuclease A, and human hemoglobin were incubated with ribose at 37°C in 0.4 M sodium phosphate buffer of pH 7.5 containing 0.02% sodium azide. The protein (10 mg/ml or 1 mg/ml), 0.05 M ribose, and prospective inhibitors (at 0.5, 3, 15 and 50 mM) were introduced into the incubation mixture simultaneously. Solutions were kept in the dark in capped tubes. Aliquots were taken and immediately frozen until analyzed by ELISA at the conclusion of the reaction. The incubations were for 3 weeks (Hb) or 6 weeks (RNase, BSA). Glycation reactions were monitored for constant pH throughout the duration of the experiments.

Interrupted (post-Amadori) ribose glycation assays
Glycation was first carried out by incubating protein (10 mg/ml) with 0.5 M ribose at 37°C in 0.4 M phosphate buffer at pH 7.5 containing 0.2% sodium azide for 24 h in the absence of inhibitors. Glycation was then interrupted to remove excess and reversibly bound (Schiff base) sugar by extensive dialysis against frequent cold buffer changes at 4°C. The glycated intermediate samples containing maximal amount of Amadori product and little AGE (depending on protein) were then quickly warmed to 37°C without re-addition of ribose. This initiated conversion of Amadori intermediates to AGE products in the absence or presence of various concentrations (typically 3, 15 and 50 mM) of

prospective inhibitors. Aliquots were taken and frozen at various intervals for later analysis. The solutions were kept in capped tubes and opened only to remove timed aliquots that were immediately frozen for later carrying out the various analyses.

Numerical Analysis of kinetics data Kinetics data (time progress curves) was routinely fit to mono- or bi-exponential functions using non-linear least squares methods utilizing either SCIENTIST 2.0 (MicroMath, Inc.) or ORIGIN (Microcal, Inc.) software that permit user-defined functions and control of parameters to iterate on. Standard deviations of the parameters of the fitted functions (initial and final ordinate values and rate constants) were returned as measures of the precision of the fits. Apparent half-times for bi-exponential kinetics fits were determined with the "solve" function of MathCad software (MathSoft, Inc.).

RESULTS

Inhibition by vitamin B_6 derivatives of the overall kinetics of AGE formation from Ribose.

The inhibitory effects of vitamin B_1 and B_6 derivatives on the kinetics of antigenic AGE formation were evaluated by polyclonal antibodies specific for AGEs. Initial inhibition studies were carried out on the glycation of bovine ribonuclease A (RNase) in the continuous presence of 0.05 M ribose, which is the concentration of ribose where the rate of AGE formation is near maximal. Figure 13 (control curves, filled rectangles) demonstrates that the formation of antigenic AGEs on RNase when incubated with 0.05 M ribose is relatively rapid, with a half-time of approximately 6 days under these conditions. Pyridoxal-5'-phosphate (Figure 13B) and pyridoxal (Figure 13C) significantly inhibited the rate of AGE formation on RNase at concentrations of 50 mM and 15 mM. Surprisingly, pyridoxine, the alcohol form of vitamin B_6, also moderately inhibited AGE formation on RNase (Figure 13D). Of the B_6 derivatives examined, pyridoxamine at 50 mM

was the best inhibitor of the "final" levels of AGE formed on RNase over the 6-week time period monitored (Figure 13A).

Inhibition by vitamin B_1 derivatives of the overall kinetics of AGE formation from Ribose.

All of the B_1 vitamers inhibited antigenic AGE formation on RNase at high concentrations, but the inhibition appeared more complex than for the B_6 derivatives (Figure 14A-C). In the case of thiamine pyrophosphate as the inhibitor (Figure 14A), both the rate of AGE formation and the final levels of AGE produced at the plateau appeared diminished. In the case of thiamine phosphate as the inhibitor (Figure 14B), and thiamine (Figure 14C), there appeared to be little effect on the rate of AGE formation, but a substantial decrease in the final level of AGE formed in the presence of the highest concentration of inhibitor. In general, thiamine pyrophosphate demonstrated greater inhibition than the other two compounds, at the lower concentrations examined.

Inhibition by aminoguanidine of the overall kinetics of AGE formation from Ribose

Inhibition of AGE formation by aminoguanidine (Figure 14D) was distinctly different from that seen in the B_1 and B_6 experiments. Increasing concentration of aminoguanidine decreased the rate of AGE formation on RNase, but did not reduce the final level of AGE formed. The final level of AGE formed after the 6-weeks was nearly identical to that of the control for all tested concentrations of aminoguanidine.

Inhibition of the overall kinetics of AGE formation in serum albumin and hemoglobin from Ribose

Comparative studies were carried out with BSA and human methemoglobin (Hb) to determine whether the observed inhibition was protein-specific. The different derivatives of vitamin B_6 (Figure 15A-D) and vitamin B_1 (Figure 16A-C) exhibited similar inhibition trends when incubated with BSA

as with RNase, pyridoxamine and thiamine pyrophosphate being the most effective inhibitors or each family. Pyridoxine failed to inhibit AGE formation on BSA (Figure 15D). Pyridoxal phosphate and pyridoxal (Figure 15B-C) mostly inhibited the rate of AGE formation, but not the final levels of AGE formed. Pyridoxamine (Figure 15A) exhibited some inhibition at lower concentrations, and at the highest concentration tested appeared to inhibit the final levels of AGE formed more effectively than any of the other B_6 derivatives. In the case of B_1 derivatives, the overall extent of inhibition of AGE formation with BSA (Figure 16A-C), was less than that observed with RNase (Figure 14A-C). Higher concentrations of thiamine and thiamine pyrophosphate inhibited the final levels of AGEs formed, without greatly affecting the rate of AGE formation (Figure 16C). Aminoguanidine again displayed the same inhibition effects with BSA as seen with RNase (Figure 16D), appearing to slow the rate of AGE formation without significantly affecting the final levels of AGE formed.

The kinetics of AGE formation was also examined using Hb in the presence of the B_6 and B_1 vitamin derivatives, and aminoguanidine. The apparent absolute rates of AGE formation were significantly higher with Hb than with either RNase or BSA. However, the tested inhibitors showed essentially similar behavior. The effects of the vitamin B_6 derivatives are shown in Figure 17. Pyridoxamine showed the greatest inhibition at concentrations of 3 mM and above (Figure 17A), and was most effective when compared to pyridoxal phosphate (Figure 17B), pyridoxal (Figure 17C), and pyridoxine (Figure 17D). In the case of the B_1 vitamin derivatives (data not shown), the inhibitory effects were more similar to the BSA inhibition trends than to RNase. The inhibition was only modest at the highest concentrations tested (50 mM), being nearly 30-50% for all three B_1 derivatives. The primary manifestation of inhibition was in the reduction of the final levels of AGE formed.

Inhibition by vitamin B_6 derivatives of the kinetics of post-Amadori ribose AGE formation

Using the interrupted glycation model to assay for inhibition of the "late" post-Amadori AGE formation, kinetics were examined by incubating isolated Amadori intermediates of either RNase or BSA at 37°C in the absence of free or reversibly bound ribose. Ribose sugar that was initially used to prepare the intermediates was removed by cold dialysis after an initial glycation reaction period of 24 h. After AGE formation is allowed to resume, formation of AGE is quite rapid (half-times of about 10 h) in the absence of any inhibitors. Figure 18 shows the effects of pyridoxamine (Figure 18A), pyridoxal phosphate (Figure 18B), and pyridoxal (Figure 18C) on the post-Amadori kinetics of BSA. Pyridoxine did not produce any inhibition (data not shown). Similar experiments were carried out on RNase. Pyridoxamine caused nearly complete inhibition of AGE formation with RNase at 15 mM and 50 mM (Figure 18D). Pyridoxal did not show any significant inhibition at 15 mM (the highest tested), but pyridoxal phosphate showed significant inhibition at 15 mM. Pyridoxal phosphate is known to be able to affinity label the active site of RNase (Raetz and Auld, 1972, *Biochemistry* **11**:2229-2236).

With BSA, unlike RNase, a significant amount of antigenic AGE formed during the 24 h initial incubation with RNase (25-30%), as evidenced by the higher ELISA readings after removal of ribose at time zero for Figures 18A-C. For both BSA and RNase, the inhibition, when seen, appears to manifest as a decrease in the final levels of AGE formed rather than as a decrease in the rate of formation of AGE.

Inhibition by vitamin B_1 derivatives of the kinetics of post-Amadori ribose AGE formation

Thiamine pyrophosphate inhibited AGE formation more effectively than the other B_1 derivatives with both RNase and BSA (Figure 19). Thiamine showed no effect, while thiamine phosphate showed some intermediate effect. As with

the B_6 assays, the post-Amadori inhibition was most apparently manifested as a decrease in the final levels of AGE formed.

Effects of aminoguanidine and N^{α}-acetyl-L-lysine on the kinetics of post-Amadori ribose AGE formation

Figure 20 shows the results of testing aminoguanidine for inhibition of post-Amadori AGE formation kinetics with both BSA and RNase. At 50 mM, inhibition was about 20% in the case of BSA (Figure 20B), and less than 15% with RNase (Figure 20A). The possibility of inhibition by simple amino-containing functionalities was also tested using N^{α}-acetyl-L-lysine (Figure 21), which contains only a free N^{ϵ}-amino group. N^{α}-acetyl-L-lysine at up to 50 mM failed to exhibit any significant inhibition of AGE formation.

Discussion

Numerous studies have demonstrated that aminoguanidine is an apparently potent inhibitor of many manifestations of nonenzymatic glycation (Brownlee et al., 1986; Brownlee, 1992,1994, 1995). The inhibitory effects of aminoguanidine on various phenomena that are induced by reducing sugars are widely considered as proof of the involvement of glycation in many such phenomena. Aminoguanidine has recently entered into a second round of Phase III clinical trials (as pimagedine) for ameliorating the complications of diabetes thought to be caused by glycation of connective tissue proteins due to high levels of sugar.

Data from the kinetic study of uninterrupted "slow" AGE formation with RNase induced by glucose (Example 1) confirmed that aminóguanidine is an effective inhibitor, and further identified a number of derivatives of vitamins B_1 and B_6 as equally or slightly more effective inhibitors. However, the inhibition by aminoguanidine unexpectedly appeared to diminish in effect at the later stages of the AGE formation reaction. Due to the slowness of the glycation of protein with glucose, this surprising observation could

not be fully examined. Furthermore, it has been suggested that there may be questions about the long-term stability of aminoguanidine (Ou and Wolff, 1993, *supra*).

Analysis using the much more rapid glycation by ribose allowed for the entire time-course of AGE formation to be completely observed and quantitated during uninterrupted glycation of protein. The use of interrupted glycation uniquely allowed further isolation and examination of only post-Amadori antigenic AGE formation in the absence of free and reversibly bound (Schiff base) ribose. Comparison of the data from these two approaches with the earlier glucose glycation kinetics has provided novel insights into the mechanisms and effectiveness of various inhibitors. Figure 22 are bar graphs which depict summarized comparative data of percent inhibition at defined time points using various concentrations of inhibitor. Figure 22A graphs the data for inhibition after interrupted glycation of RNase AGE formation in ribose. Figure 22B graphs the data for inhibition after interrupted glycation of BSA AGE formation by ribose.

The overall results unambiguously demonstrate that aminoguanidine slows the rate of post-Amadori antigenic AGE formation in the presence of sugar but has little effect on the final amount of post-Amadori AGE formed. Thus observations limited to only the initial "early" stages of AGE formation which indicate efficacy as an inhibitor may in fact be misleading as to the true efficacy of inhibition of post-Amadori AGE formation. Thus the ability to observe a full-course of reaction using ribose and interrupted glycation gives a more complete picture of the efficacy of inhibition of post-Amadori AGE formation.

Example 4

Animal model & testing of in vivo effects of AGE formation/inhibitors

Hyperglycemia can be rapidly induced (within one or two days) in rats by administration of streptozocin (aka.

streptozotocin, STZ) or alloxan. This has become a common model for diabetes melitus. However, these rats manifest nephropathy only after many months of hyperglycemia, and usually just prior to death from end-stage renal disease (ESRD). It is believed that this pathology is caused by the irreversible glucose chemical modification of long-lived proteins such as collagen of the basement membrane. STZ-diabetic rats show albuminuria very late after induction of hyperglycemia, at about 40 weeks usually only just prior to death.

Because of the dramatic rapid effects of ribose demonstrated *in vitro* in the examples above, it was undertaken to examine the effects of ribose administration to rats, and the possible induction of AGEs by the rapid ribose glycation. From this study, a rat model for accelerated ribose induced pathology has been developed.

Effects of very short-term ribose administration in vivo

Phase I Protocol

Two groups of six rats each were given in one day either:

a. 300 mM ribose (two intraperitoneal infusions 6-8 hours apart, each 5% of body weight as ml); or

b. 50 mM ribose (one infusion)

Rats were then kept for 4 days with no further ribose administration, at which time they were sacrificed and the following physiological measurements were determined: (i) initial and final body weight; (ii) final stage kidney weight; (iii) initial and final tail-cuff blood pressure; (iv) creatinine clearance per 100 g body weight.

Albumin filtration rates were not measured, since no rapid changes were initially anticipated. Past experience with STZ-diabetic rats shows that albuminuria develops very late (perhaps 40 weeks) after the induction of hyperglycemia and just before animals expire.

Renal Physiology Results

a. Final body weight and final single kidney weight was same for low and high ribose treatment groups.

b. Tail-cuff blood pressure increased from 66 ± 4 to 83 ± 3 to rats treated with low ribose (1 x 50 mM), and from 66 ± 4 to 106 ± 5 for rats treated with high ribose (2 x 300 mM). These results are shown in the bar graph of Figure 23.

c. Creatinine clearance, as cc per 100 g body weight, was decreased (normal range expected about 1.0-1.2) in a dose-dependent fashion to 0.87 ± 0.15 for the low ribose group, and decreased still further 30% to 0.62 ± 0.13 for the high ribose group. These results are shown in the bar graph of Figure 24.

Phase I Conclusion

A single day's ribose treatment caused a dose-dependent hypertension and a dose-dependent decrease in glomerular clearance function manifest 4 days later. These are significant metabolic changes of diabetes seen only much later in STZ-diabetic rats. These phenomenon can be hypothesized to be due to ribose irreversible chemical modification (glycation) of protein *in vivo*.

Effect of exposure to higher ribose concentrations for longer time

Phase II Protocol

Groups of rats (3-6) were intraperitoneally given 0.3 M "low ribose dose" (LR) or 1.0 M "high ribose dose" (HR) by twice-daily injections for either (i) 1 day, (ii) a "short-term" (S) of 4 days, or (iii) a "long-term" (L) of 8 days. Additionally, these concentrations of ribose were included in drinking water.

Renal Physiology Results

a. Tail-cuff blood pressure increased in all groups of ribose-treated rats, confirming Phase I results. (Figure 23).

b. Creatinine clearance decreased in all groups in a ribose dose-dependent and time-dependent manner (Figure 24).

c. Albumin Effusion Rate (AER) increased significantly in a ribose-dependent manner at 1-day and 4-day exposures. However, it showed some recovery at 8 day relative to 4 day in the high-dose group but not in the low-dose group. These results are shown in the bar graph of Figure 25.

d. Creatinine clearance per 100 g body weight decreased for both low- and high-ribose groups to about the same extent in a time-dependent manner (Figure 24).

Phase II Conclusion

Exposure to ribose for as little as 4 days leads to hypertension and renal dysfunction, as manifest by both decreased creatinine clearance and increased albumin filtration. These changes are typical of diabetes and are seen at much later times in STZ-diabetic rats.

Intervention by two new therapeutic compounds and aminoguanidine

Phase III Protocol

Sixty rats were randomized into 9 different groups, including those exposed to 1 M ribose for 8 days in the presence and absence of aminoguanidine, pyridoxamine, and thiamine pyrophosphate as follows:

Control Groups:

(i) no treatment;

(ii) high dose (250 mg/kg body weight) of pyridoxamine ("compound-P");

(iii) high dose (250 mg/kg body weight of thiamine pyrophosphate ("compound-T" or "TPP"); and

(iv) low dose (25 mg/kg body weight) of aminoguanidine ("AG").

Test Groups:

(i) only 1 M ribose-saline (2 x 9 cc daily IP for 8 days);

(ii) ribose plus low dose ("LP") of pyridoxamine (25 mg/kg body weight injected as 0.5 ml with 9 cc ribose);

(iii) ribose plus high dose ("HP") of pyridoxamine (250 mg/kg body weight injected as 0.5 ml with 9 cc ribose);
(iv) ribose plus high dose ("HT") of thiamine pyrophosphate (250 mg/kg body weight injected as 0.5 ml with 9 cc ribose); and
(v) ribose plus low dose of amino guanidine (25 mg/kg body weight injected as 0.5 ml with 9 cc ribose).

Unlike Phase II, no ribose was administered in drinking water. Intervention compounds were pre-administered for one day prior to introducing them with ribose.

Renal physiology Results

a. Blood pressure was very slightly increased by the three compounds alone (control group); ribose-elevated BP was not ameliorated by the co-administration of compounds. These results are shown in the bar graph of Figure 26.

b. Creatinine clearance in controls was unchanged, except for TPP which diminished it.

c. Creatinine clearance was normalized when ribose was co-administerd with low dose (25 mg/kg) of either aminoguanidine or pyridoxamine. These results are shown in the bar graph of Figure 27.

d. High concentrations (250 mg/kg) or pyridoxamine and TPP showed only partial protection against the ribose-induced decrease in creatinine clearance (Figure 27).

e. Albumin effusion rate (AER) was elevated by ribose, as well as by high dose of pyridoxamine and TPP, and low dose of aminoguanidine in the absence of ribose. These results are shown in the bar graph of Figure 28.

f. Albumin effusion rate was restored to normal by the co-administration of low dose of both aminoguanidine and pyridoxamine. These results are shown in the bar graph of Figure 29.

Phase III Conclusions

As measured by two indicies of renal function, pyridoxamine and aminoguanidine, both at 25 mg/kg, were

apparently effective, and equally so, in preventing the ribose-induced decrease in creatinine clearance and ribose-induced mild increase in albuminuria.

(i) Thiamine pyrophosphate was not tested at 25 mg/kg; (ii) thiamine pyrophosphate and pyridoxamine at 250 mg/kg were partially effective in preventing creatinine clearance decreases but possibly not in preventing mild proteinuria; (iii) at these very high concentrations and in the absence of ribose, thiamine pyrophosphate alone produced a decrease in creatinine clearance, and both produced mild increases in albuminuria.

Summary

Renal Function and Diabetes

Persistent hyperglycemia in diabetes mellitus leads to diabetic nephropathy in perhaps one third of human patients. Clinically, diabetic nephropathy is defined by the presence of:

1. decrease in renal function (impaired glomerular clearance)
2. an increase in urinary protein (impaired filtration)
3. the simultaneous presence of hypertension

Renal function depends on blood flow (not measured) and the glomerular clearance, which can be measured by either inulin clearance (not measured) or creatinine clearance. Glomerular permeability is measured by albumin filtration rate, but this parameter is quite variable. It is also a log-distribution function: a hundred-fold increase in albumin excretion represents only a two-fold decrease in filtration capacity.

Ribose Diabetic Rat Model

By the above criteria, ribose appears to very rapidly induce manifestations of diabetic nephropathy, as reflected in hypertension, creatinine clearance and albuminuria, even though the latter is not large. In the established STZ diabetic rat, hyperglycemia is rapidly established in 1-2

days, but clinical manifestations of diabetic nephropathy arise very late, perhaps as much as 40 weeks for albuminuria. In general, albuminuria is highly variable from day to day and from animal to animal, although unlike humans, most STZ rats do eventually develop nephropathy.

Intervention by Compounds

Using the ribose-treated animals, pyridoxamine at 25 mg/kg body weight appears to effectively prevent two of the three manifestations usually attributed to diabetes, namely the impairment of creatinine clearance and albumin filtration. It did so as effectively as aminoguanidine. The effectiveness of thiamine pyrophosphate was not manifest, but it should be emphasized that this may be due to its use at elevated concentrations of 250 mg/kg body weight. Pyridoxamine would have appeared much less effective if only the results at 250 mg/kg body weight are considered.

Effect of Compounds Alone

Overall, the rats appeared to tolerate the compounds well. Kidney weights were not remarkable and little hypertension developed. The physiological effects of the compounds were only tested at 250 mg/kg. Thiamine pyrophosphate, but not pyridoxamine, appeared to decrease creatinine clearance at this concentration. Both appeared to slightly increase albuminuria, but these measurements were perhaps the least reliable.

Human Administration

A typical adult human being of average size weighs between 66 - 77 Kg. Typically, diabetic patients may tend to be overweight and can be over 112 Kg. The Recommended dietary allowances for an adult male of between 66 - 77 Kg, as revised in 1989, called for 1.5 mg per day of thiamine, and 2.0 mg per day of Vitamin B_6 (Merck Manual of Diagnosis and Therapy, 16th edition (Merck & Co., Rathaway, N.J., 1992) pp 938-939).

Based upon the rat model approach, a range of doses for administration of pyridoxamine or thiamine pyrophosphate that is predicted to be effective for inhibiting post-Amadori AGE formation and thus inhibiting related pathologies would fall in the range of 1 mg/100 g body weight to 200 mg/100 g body weight. The appropriate range when co-administered with aminoguanidine will be similar. Calculated for an average adult of 75 Kg, the range (at 10 mg/1 Kg body weight) can be approximately 750 mg to upwards of 150 g (at 2 g/1 Kg body weight). This will naturally vary according to the particular patient.

The instant invention may be embodied in other forms or carried out in other ways without departing from the spirit or essential characteristics thereof. The present disclosure and enumerated examples are therefore to be considered as in all respects illustrative and not restrictive, the scope of the invention being indicated by the appended claims, and all equivalency are intended to be embraced therein. One of ordinary skill in the art would be able to recognize equivalent embodiments of the instant invention, and be able to practice such embodiments using the teaching of the instant disclosure and only routine experimentation.

What is claimed is:

1. A method for generating stable protein-sugar post-Amadori advanced glycation end-product intermediates comprising incubating a protein with sugar at a sufficient concentration and for sufficient length of time to generate stable post-Amadori AGE intermediates.

2. The method of claim 1 wherein the sugar is selected from the group consisting of ribose, lyxose, xylose, arabinose, glucose, fructose, maltose, lactose, mannose, fructose and galactose.

3. The method of claim 1 wherein the sugar is ribose.

4. The method of claim 2 wherein the sugar is at a concentration of 0.005 M to 10 M sugar.

5. The method of claim 1 wherein the protein is exposed to sugar from 1 hour to 60 days.

6. A method for rapidly generating antigenic post-Amadori advanced glycation endproduct comprising releasing a stable protein-sugar post-Amadori advanced glycation end-product intermediary from inhibitory conditions.

7. The method of claim 6 wherein the inhibitory conditions are selected from the group consisting of high sugar concentration, low temperature and high sugar concentration, low pH and high sugar concentration, and high pH and high sugar concentration.

8. The method of claim 6 wherein the release from inhibitory conditions is selected from the group consisting of, release by rapid dilution of sugar concentration, release by dialysis of sugar, rapid adjustment of pH.

9. The method of claim 6 wherein the sugar is a pentose sugar.

10. The method of claim 6 wherein the sugar is ribose.

11. The method of claim 10 wherein the ribose is administered at a concentration of 0.005 M to 10 M ribose sugar.

12. A method for inhibiting post-Amadori AGE formation comprising administering an effective post-Amadori AGE inhibiting amount of thiamine pyrophosphate.

13. A method for inhibiting post-Amadori AGE formation comprising administering an effective post-Amadori AGE inhibiting amount of pyridoxamine.

14. An improved method of inhibiting protein cross-linking by the administration of aminoguanidine, the improvement being administration in combination with an effective post-Amadori AGE inhibiting amount of pyridoxamine.

15. An improved method of inhibiting protein cross-linking by the administration of aminoguanidine, the improvement being administration in combination with an effective post-Amadori AGE inhibiting amount of thiamine pyrophosphate.

16. An improved method of inhibiting protein cross-linking by the administration of aminoguanidine, the improvement being administration in combination with an effective post-Amadori AGE inhibiting amount of pyridoxamine and thiamine pyrophosphate.

17. A method for rapidly generating a rat model for diabetes with end-stage post-Amadori AGE related pathology comprising administering ribose to a rat.

18. A method for identifying an effective inhibitor of the formation of late Maillard products comprising: generating stable protein-sugar post-Amadori advanced glycation end-product intermediates by incubating a protein with sugar at a sufficient concentration and for sufficient length of time to generate stable post-Amadori AGE intermediates; contacting said stable protein-sugar post-Amadori advanced glycation end-product intermediates with an inhibitor candidate; identifying effective inhibition by monitoring the formation of post-Amadori AGEs after release of the stable protein-sugar post-Amadori advanced glycation end-product intermediates from sugar induced equilibrium by assay for post-Amadori AGE formation.

19. An effective inhibitor of post-Amadori AGE formation, as characterized by the ability to inhibit the formation of post-Amadori AGE endproducts in an assay comprising: generating stable protein-sugar post-Amadori advanced glycation end-product intermediates by incubating a protein with sugar at a sufficient concentration and for sufficient length of time to generate stable post-Amadori AGE intermediates; contacting said stable protein-sugar post-Amadori advanced glycation end-product intermediates with an inhibitor candidate; identifying effective inhibition by monitoring the formation of post-Amadori AGEs after release of the stable protein-sugar post-Amadori advanced glycation end-product intermediates from sugar induced equilibrium by assay for AGE formation.

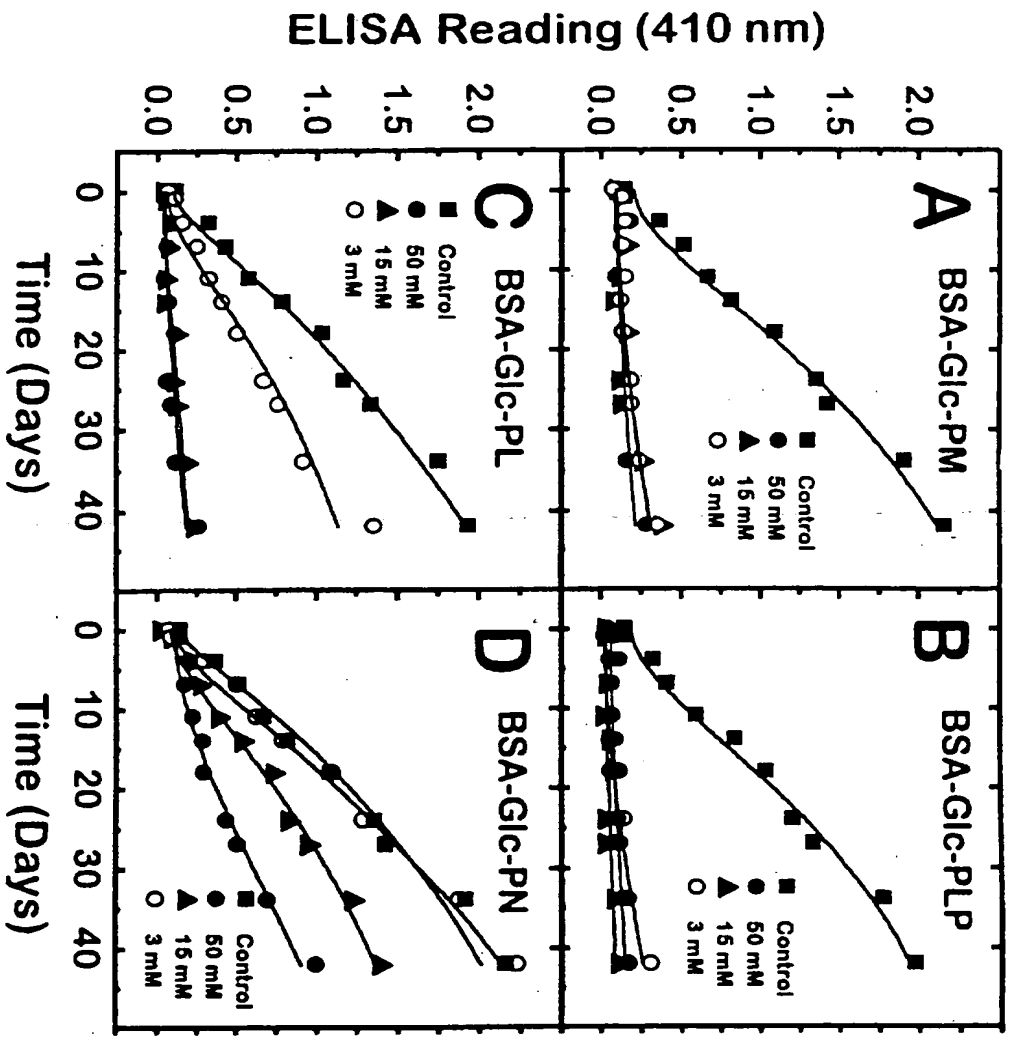

ELISA Reading (410 nm)
A BSA-Glc-PM
B BSA-Glc-PLP
C BSA-Glc-PL
D BSA-Glc-PN
Control
50 mM
15 mM
3 mM
Time (Days)
0.0
0.5
1.0
1.5
2.0
0
10
20
30
40

Fig. 1

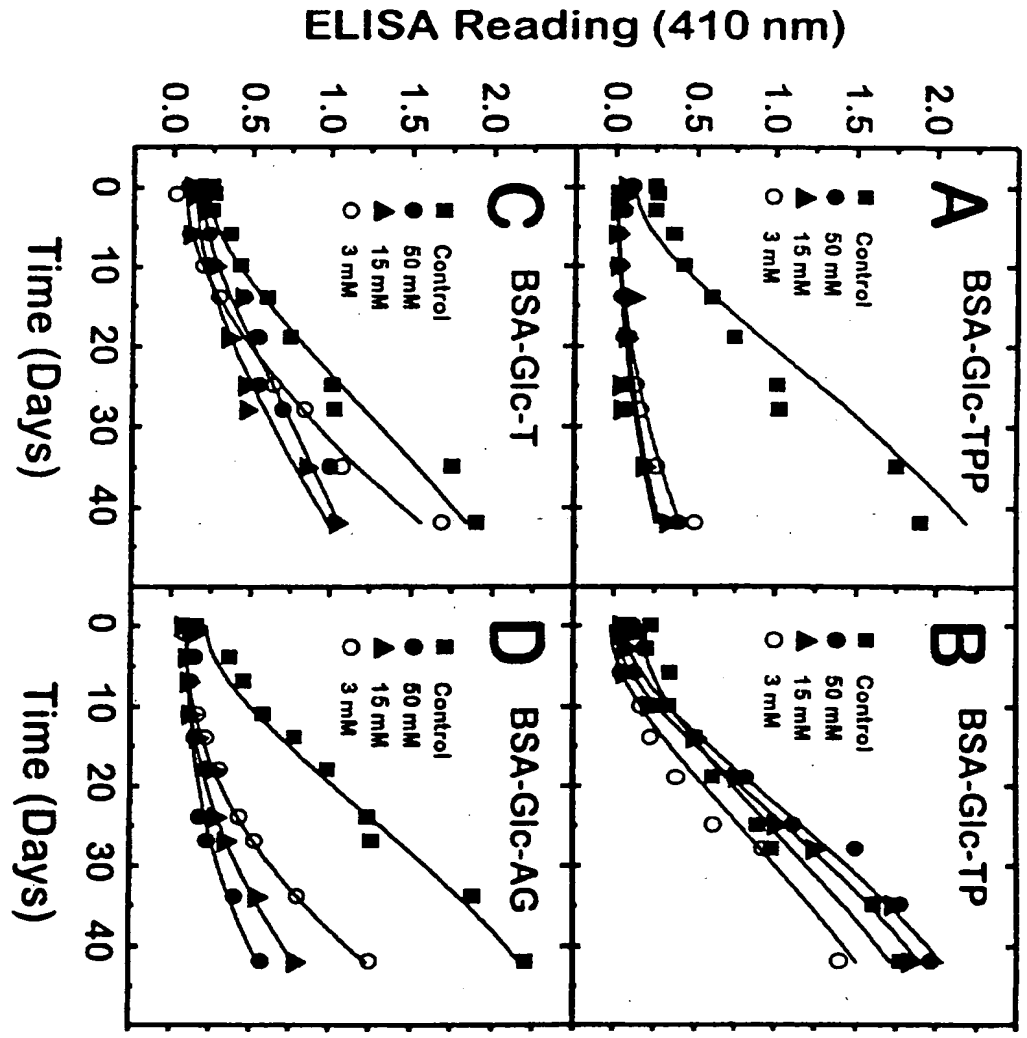

ELISA Reading (410 nm)
A BSA-Glc-TPP
B BSA-Glc-TP
C BSA-Glc-T
D BSA-Glc-AG
Control
50 mM
15 mM
3 mM
0.0
0.5
1.0
1.5
2.0
0 10 20 30 40
Time (Days)

Fig. 2

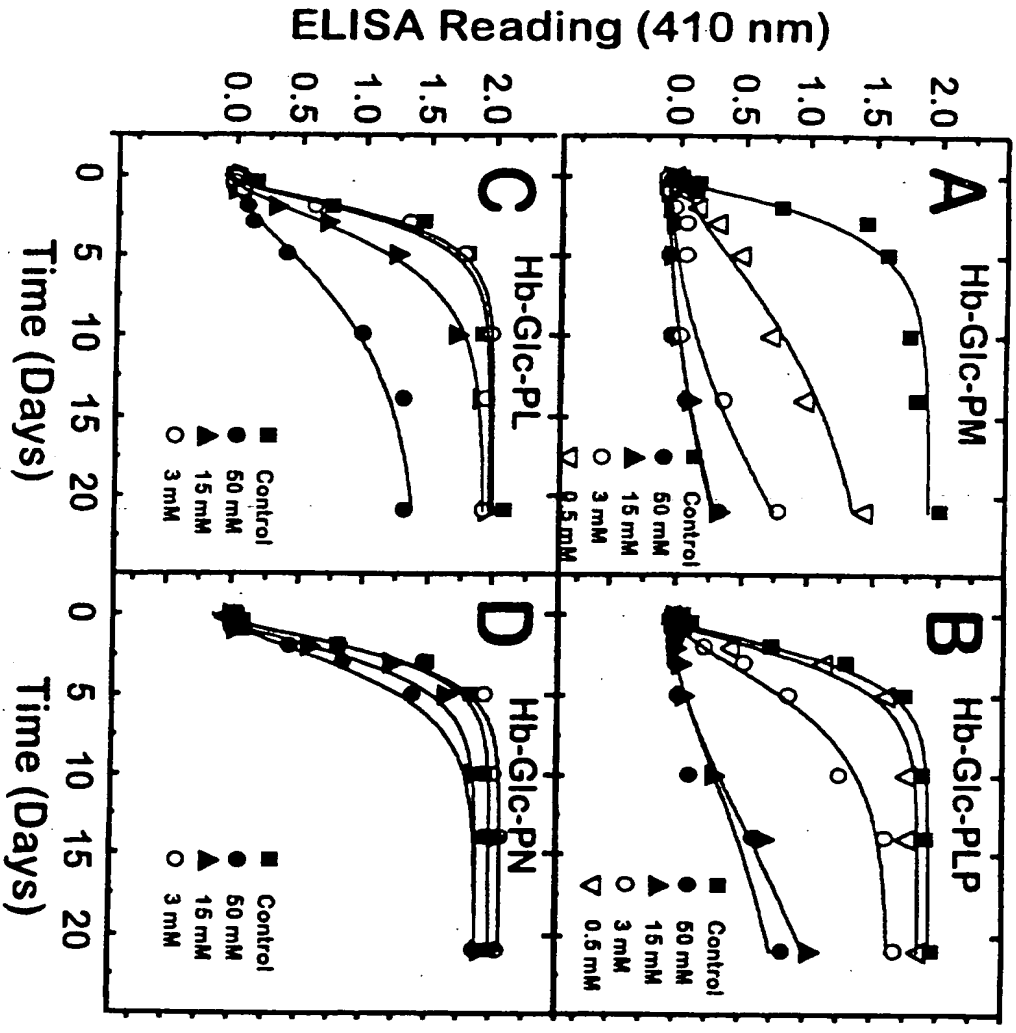

ELISA Reading (410 nm)
A
Hb-Glc-PM
B
Hb-Glc-PLP
C
Hb-Glc-PL
D
Hb-Glc-PN
Control
50 mM
15 mM
3 mM
0.5 mM
Time (Days)

Fig. 3

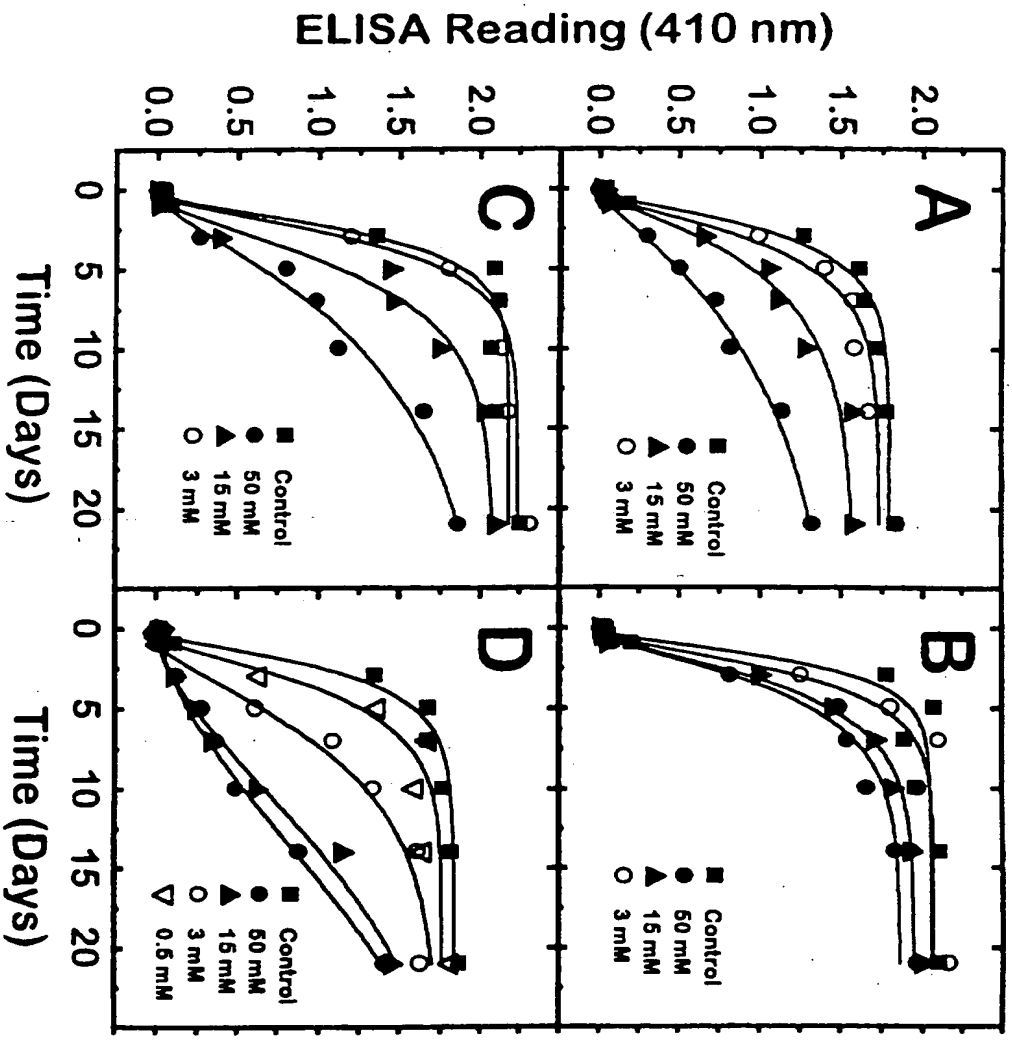

ELISA Reading (410 nm)
0.0
0.5
1.0
1.5
2.0
A
B
C
D
Control
50 mM
15 mM
3 mM
0.5 mM
0
5
10
15
20
Time (Days)

Fig. 4

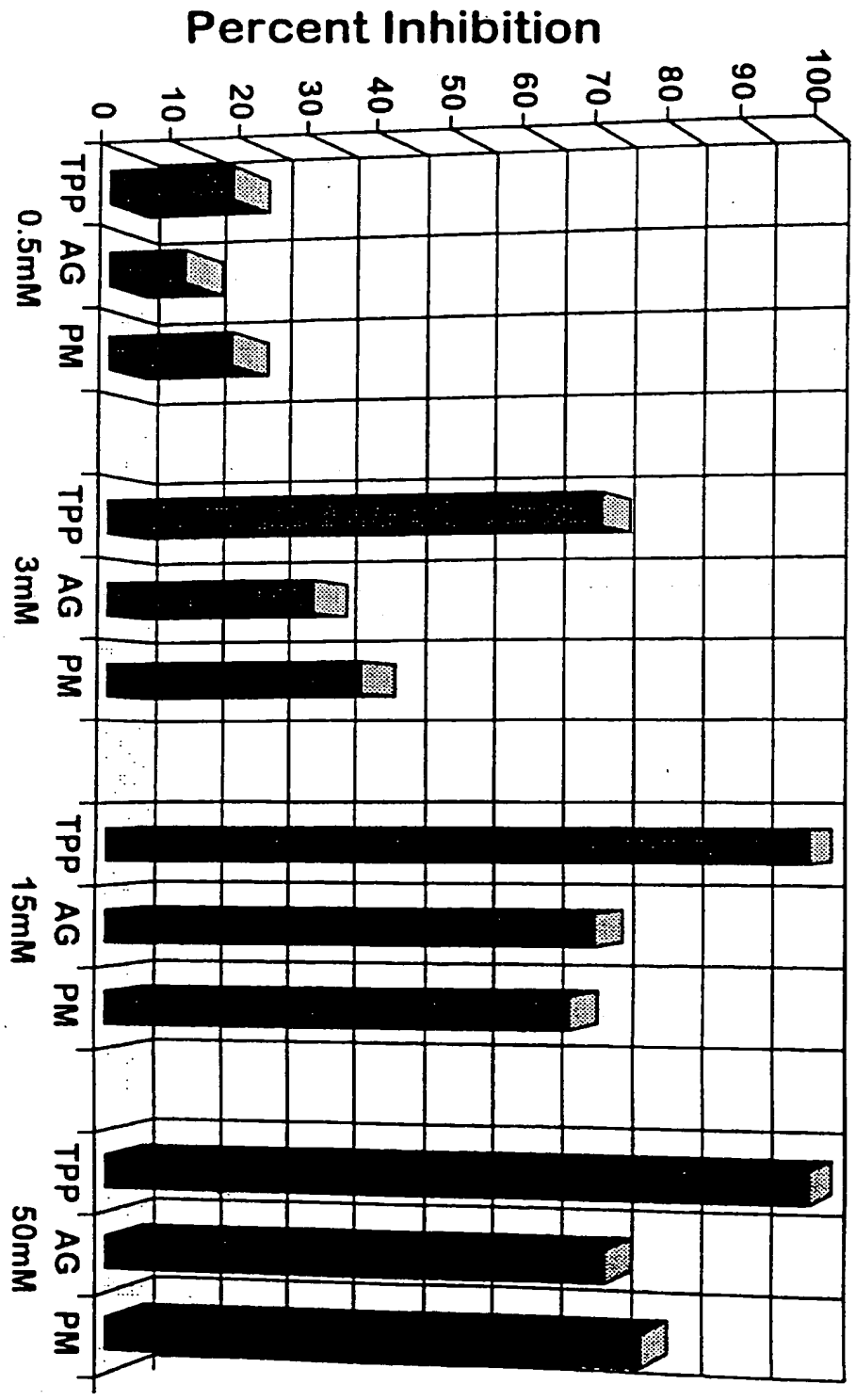
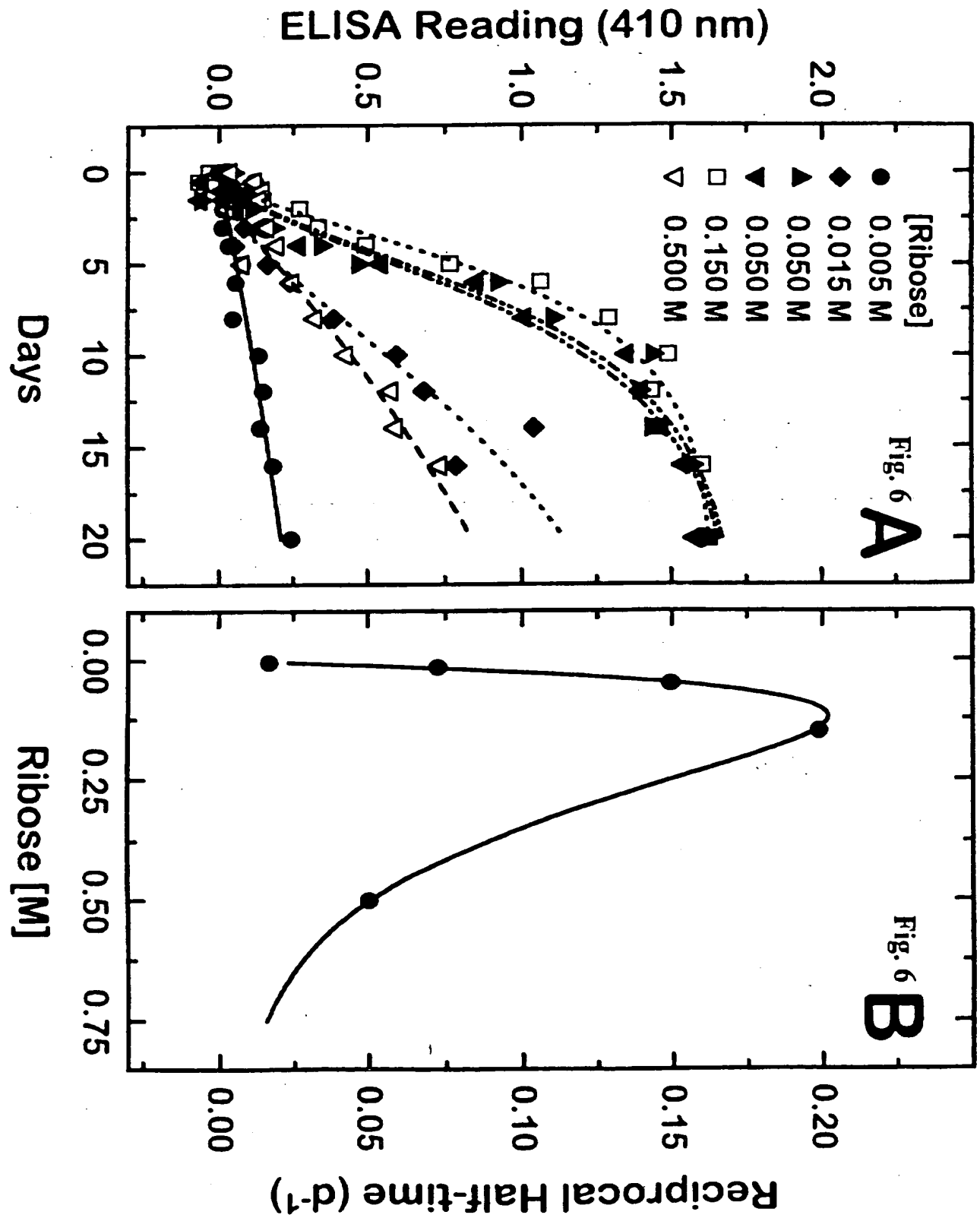

Percent Inhibition
0
10
20
30
40
50
60
70
80
90
100
TPP
AG
PM
0.5mM
3mM
15mM
50mM

Fig. 5


ELISA Reading (410 nm)
0.0
0.5
1.0
1.5
2.0
Days
0
5
10
15
20
[Ribose]
0.005 M
0.015 M
0.050 M
0.050 M
0.150 M
0.500 M
A
Fig. 6
Ribose [M]
0.00
0.25
0.50
0.75
Reciprocal Half-time (d-1)
0.00
0.05
0.10
0.15
0.20
B
Fig. 6

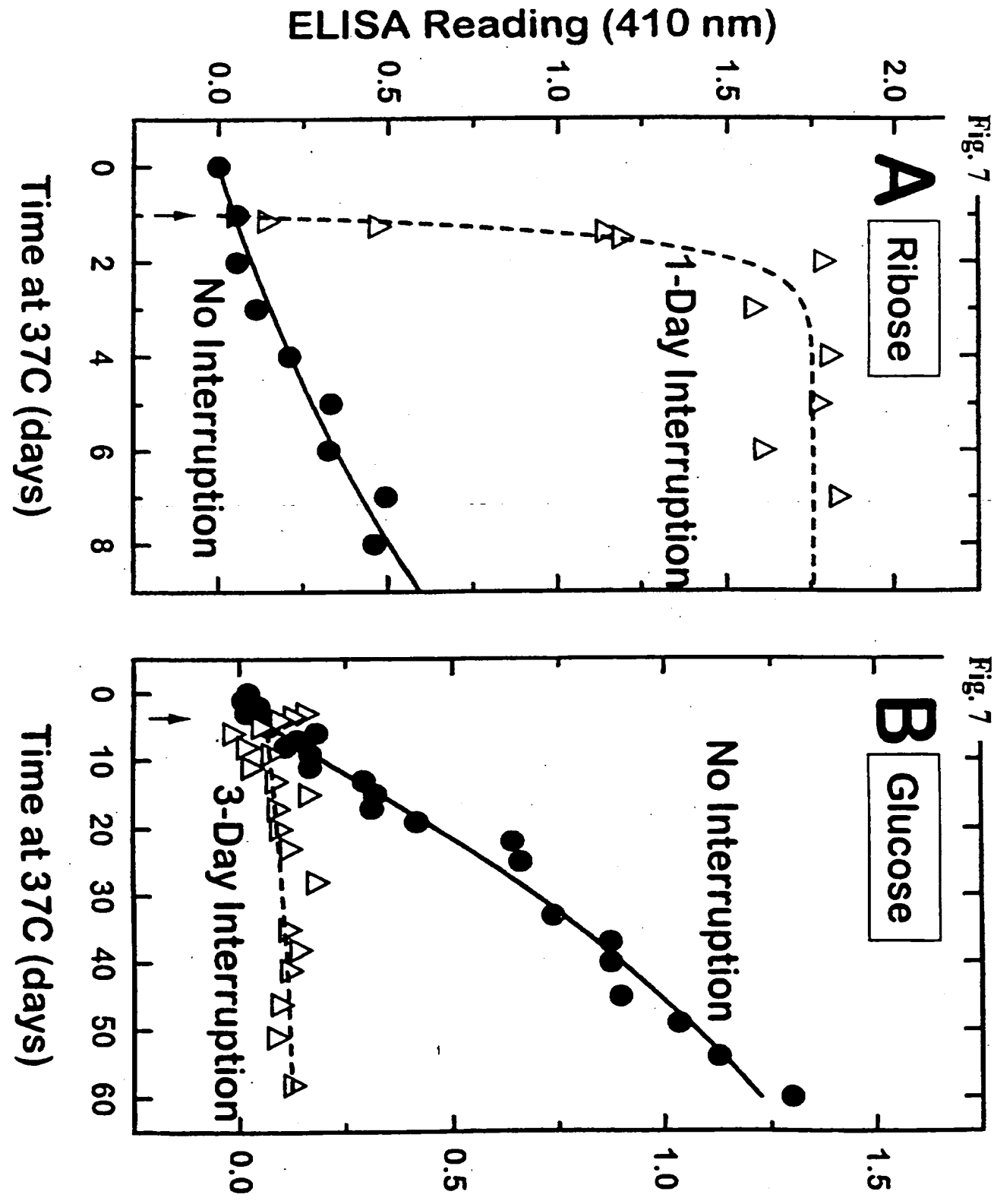

ELISA Reading (410 nm)
0.0
0.5
1.0
1.5
2.0
A
Ribose
1-Day Interruption
No Interruption
0
2
4
6
8
Time at 37C (days)
B
Glucose
No Interruption
3-Day Interruption
0.0
0.5
1.0
1.5
0
10
20
30
40
50
60
Time at 37C (days)

Fig. 7

Fig. 7

Fig. 8

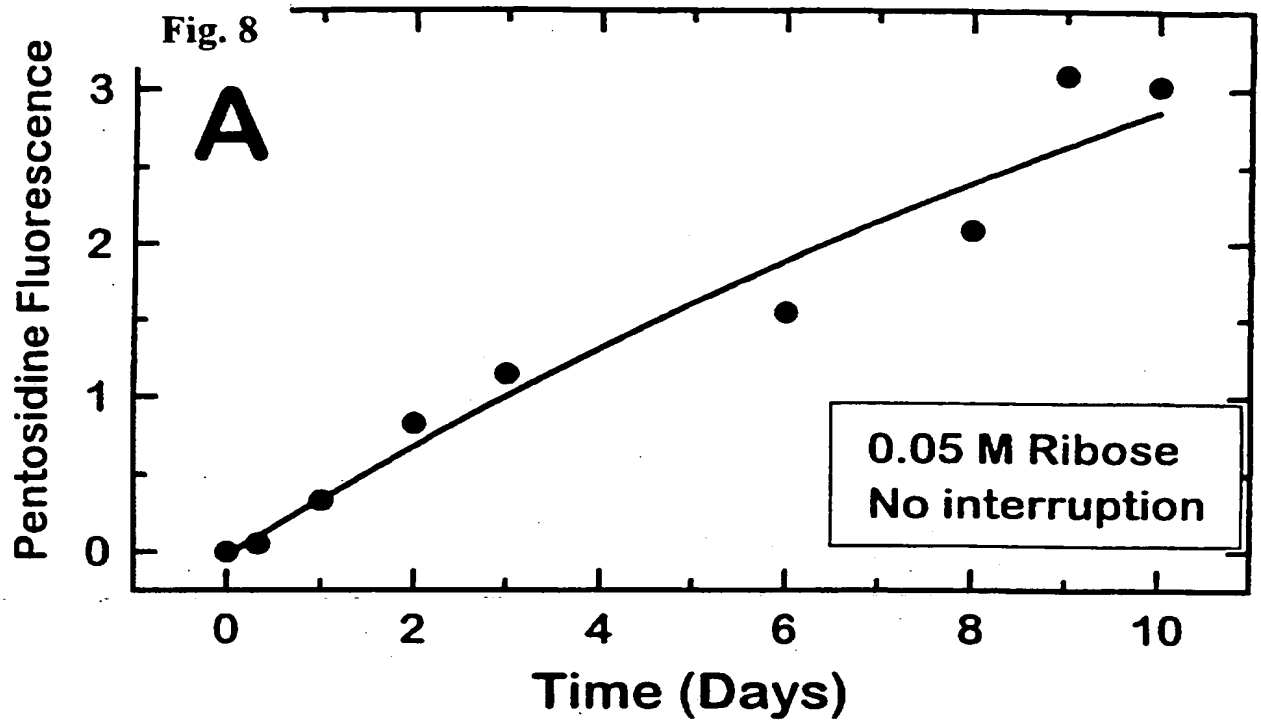
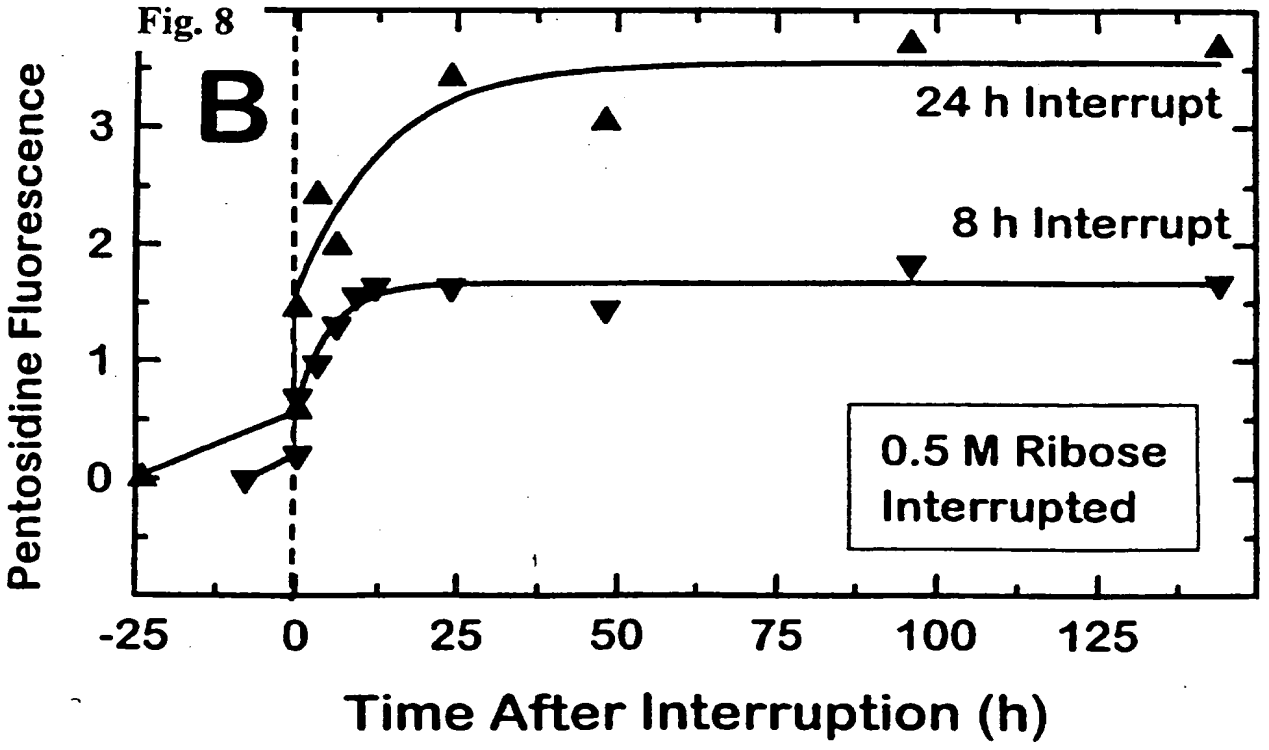

A
Pentosidine Fluorescence
0
1
2
3
0
2
4
6
8
10
Time (Days)
0.05 M Ribose
No interruption

Fig. 8


B
Pentosidine Fluorescence
0
1
2
3
-25
0
25
50
75
100
125
Time After Interruption (h)
24 h Interrupt
8 h Interrupt
0.5 M Ribose
Interrupted

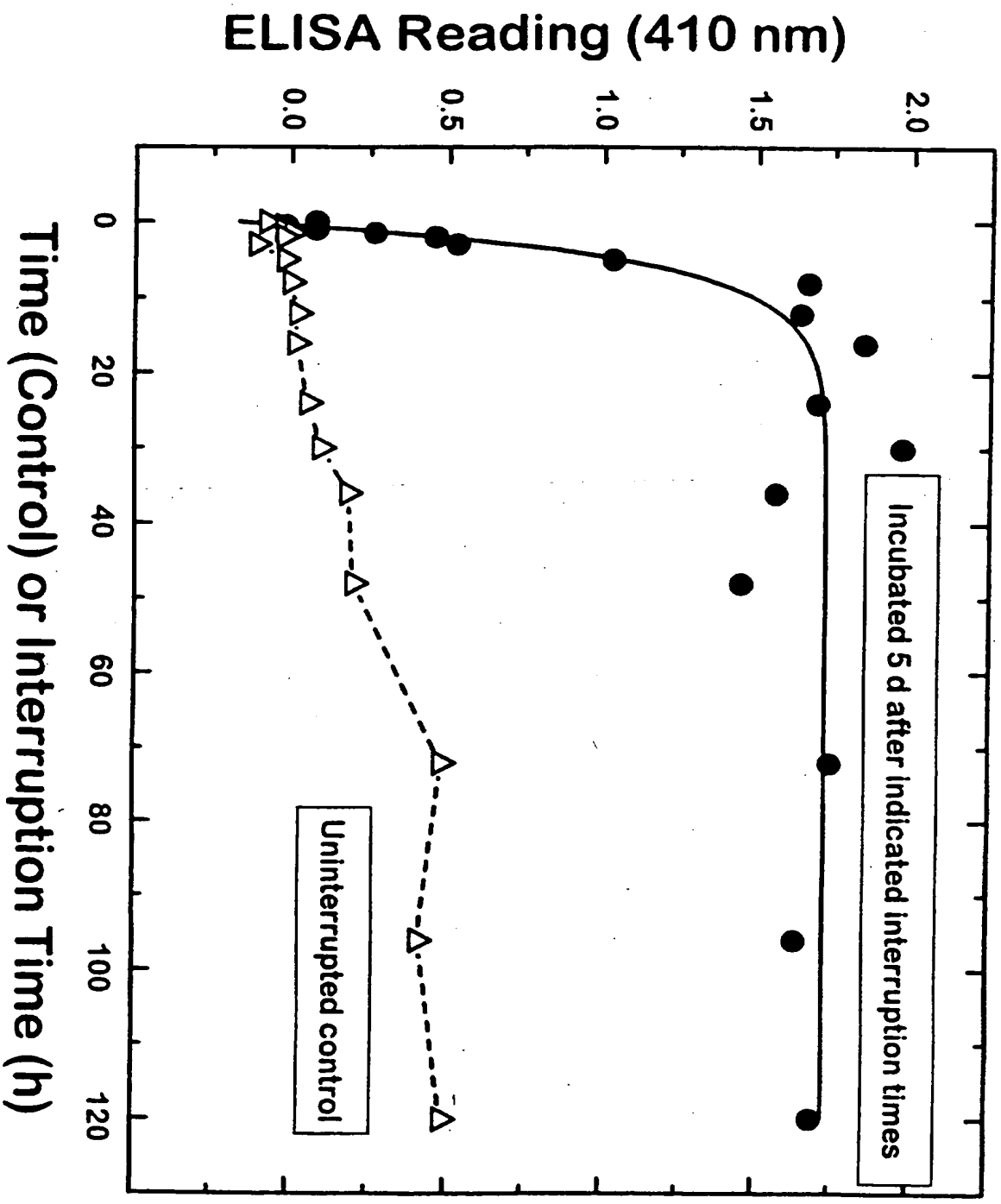
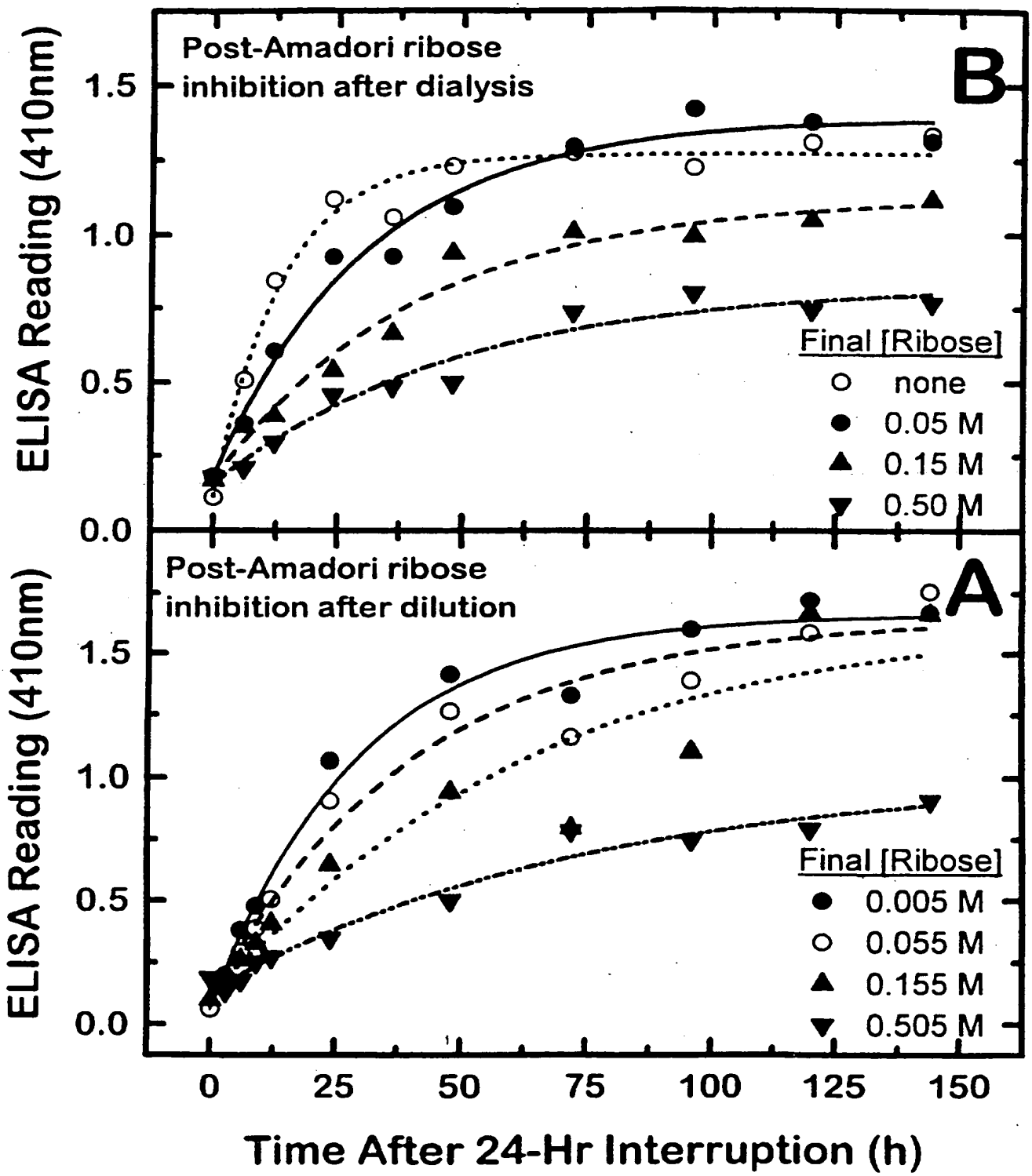

ELISA Reading (410 nm)
0.0
0.5
1.0
1.5
2.0
Time (Control) or Interruption Time (h)
0
20
40
60
80
100
120
Incubated 5 d after indicated interruption times
Uninterrupted control

Fig. 9

Fig. 10


Post-Amadori ribose
inhibition after dialysis
B
ELISA Reading (410nm)
1.5
1.0
0.5
0.0
Final [Ribose]
none
0.05 M
0.15 M
0.50 M
Post-Amadori ribose
inhibition after dilution
A
ELISA Reading (410nm)
1.5
1.0
0.5
0.0
Final [Ribose]
0.005 M
0.055 M
0.155 M
0.505 M
0
25
50
75
100
125
150
Time After 24-Hr Interruption (h)

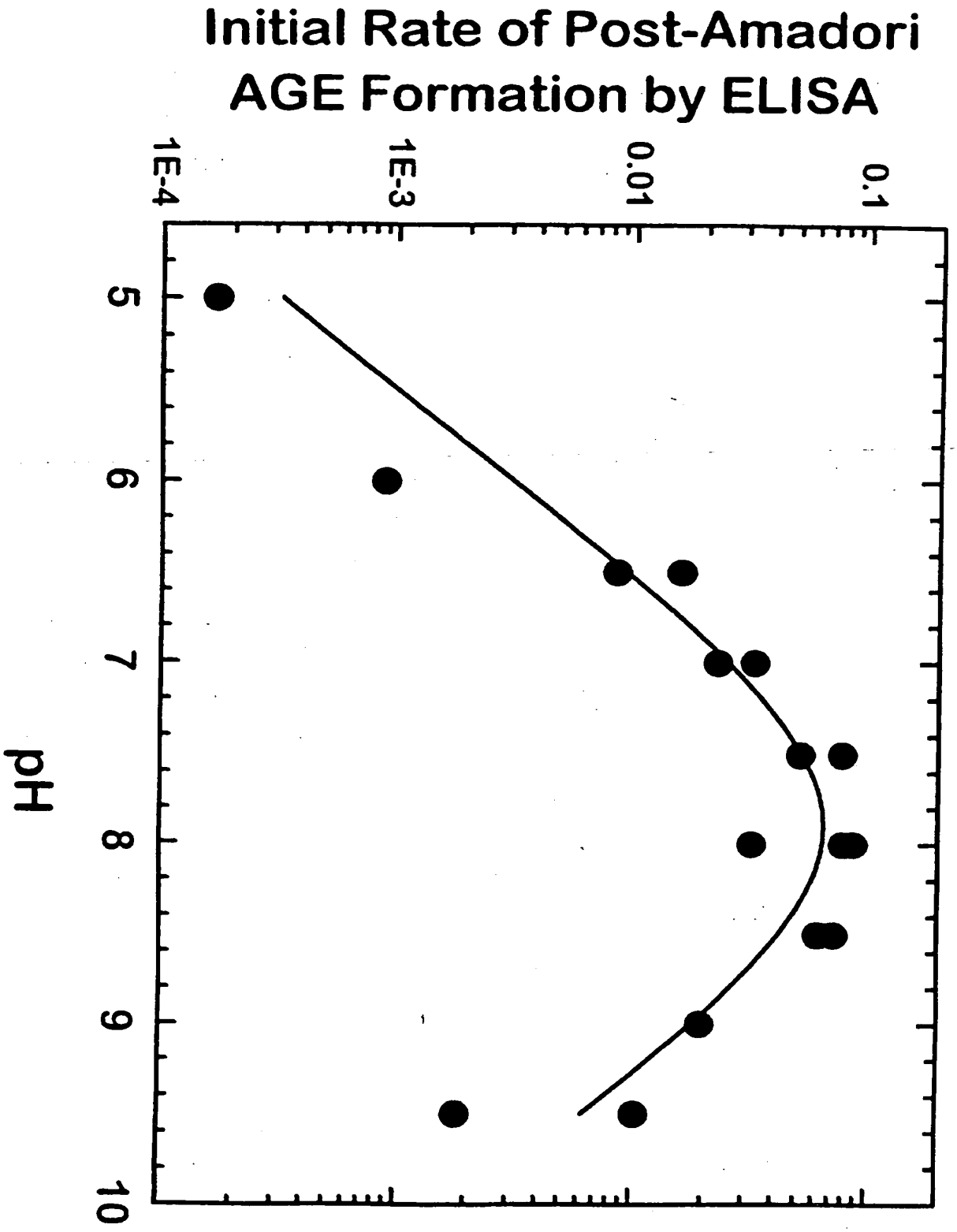
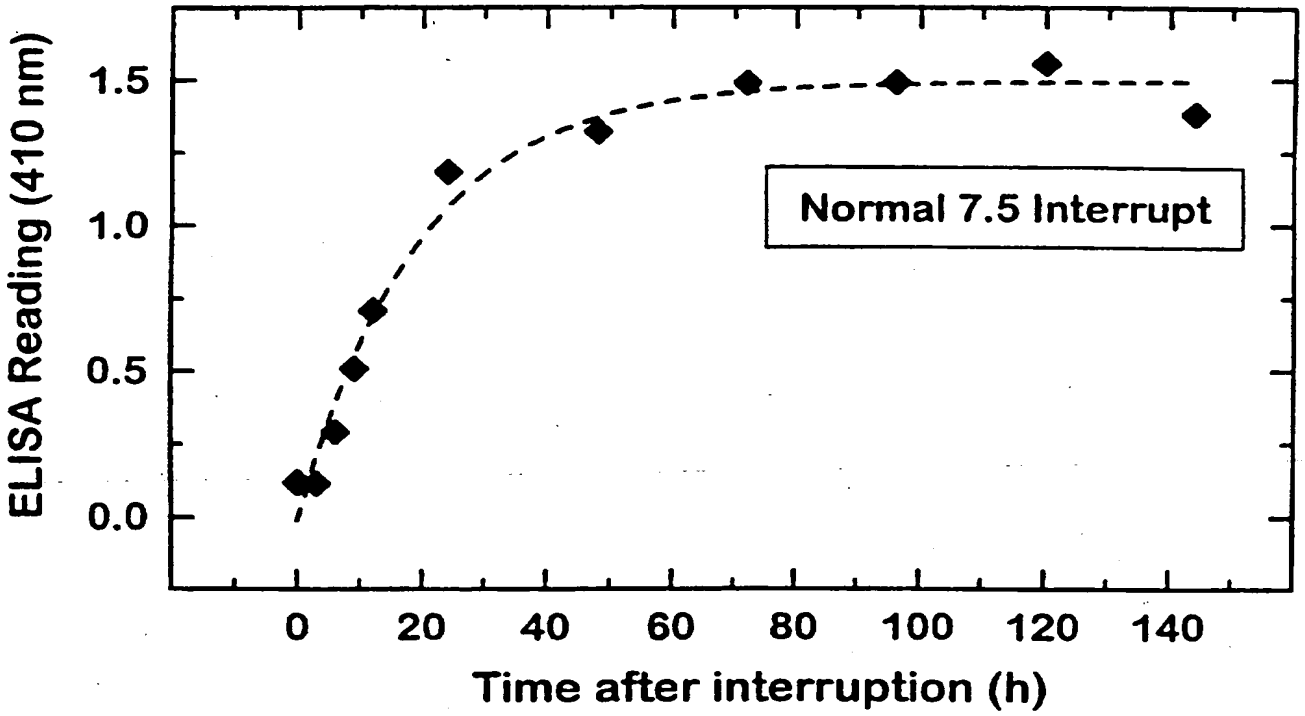

Initial Rate of Post-Amadori AGE Formation by ELISA
1E-4
1E-3
0.01
0.1
pH
5
6
7
8
9
10

Fig. 11

Fig. 12 A



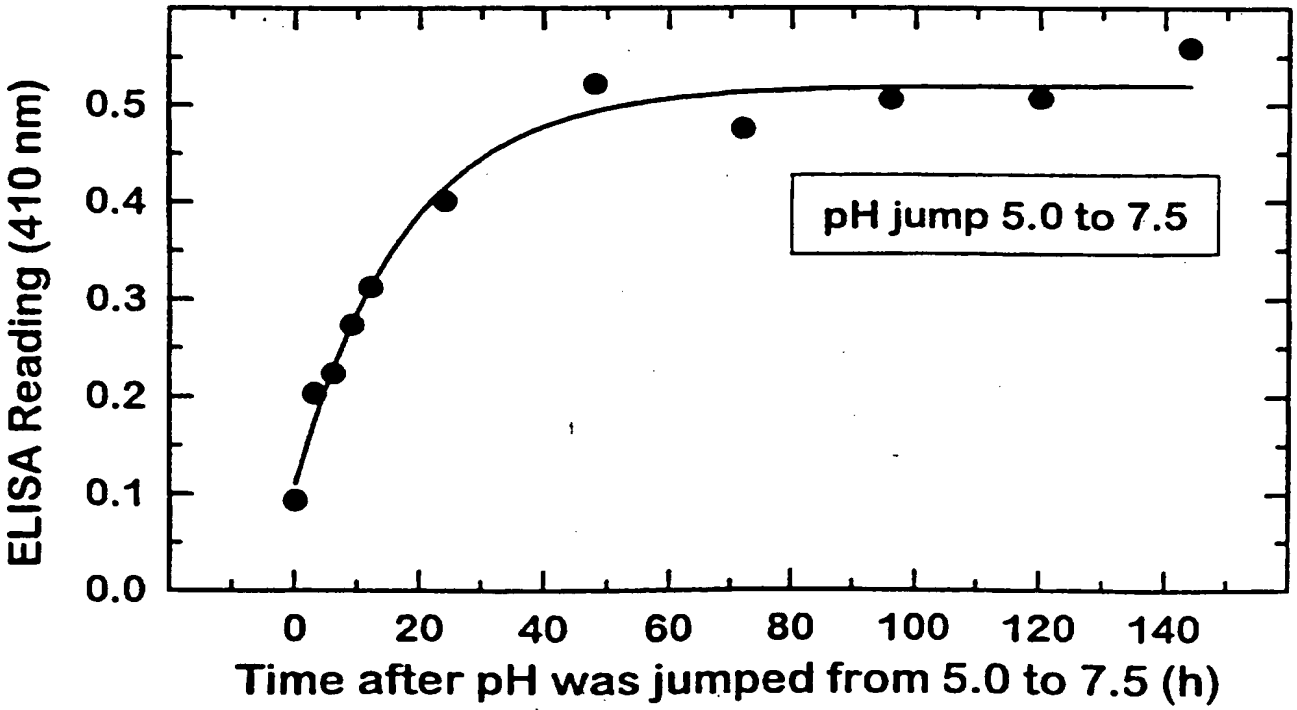


Fig. 12 B

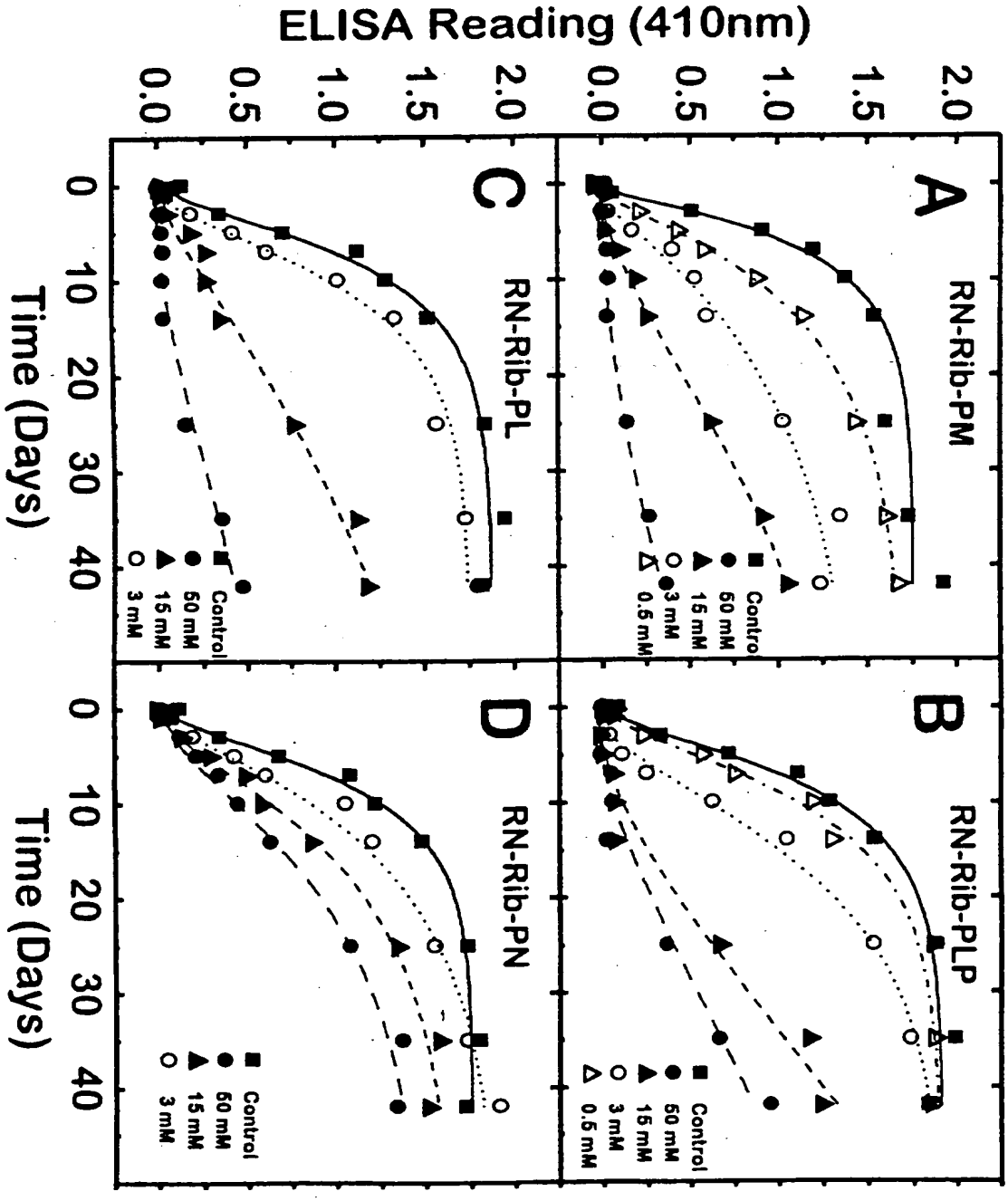

ELISA Reading (410nm)
0.0
0.5
1.0
1.5
2.0
A
RN-Rib-PM
B
RN-Rib-PLP
C
RN-Rib-PL
D
RN-Rib-PN
Control
50 mM
15 mM
3 mM
0.5 mM
0
10
20
30
40
Time (Days)

Fig. 13

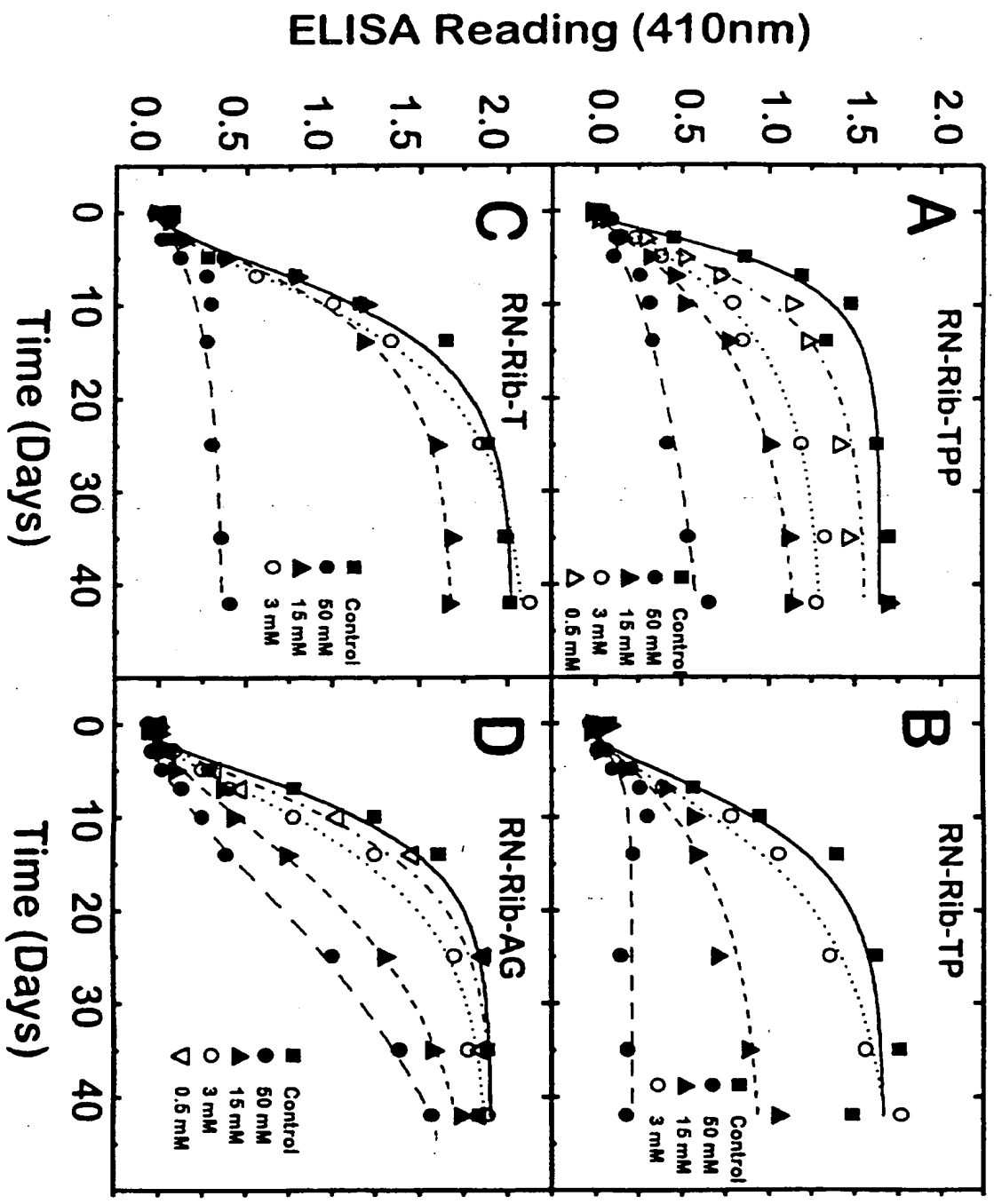

ELISA Reading (410nm)
A
RN-Rib-TPP
B
RN-Rib-TP
C
RN-Rib-T
D
RN-Rib-AG
Control
50 mM
15 mM
3 mM
0.5 mM
Time (Days)
0.0
0.5
1.0
1.5
2.0
0
10
20
30
40

Fig. 14

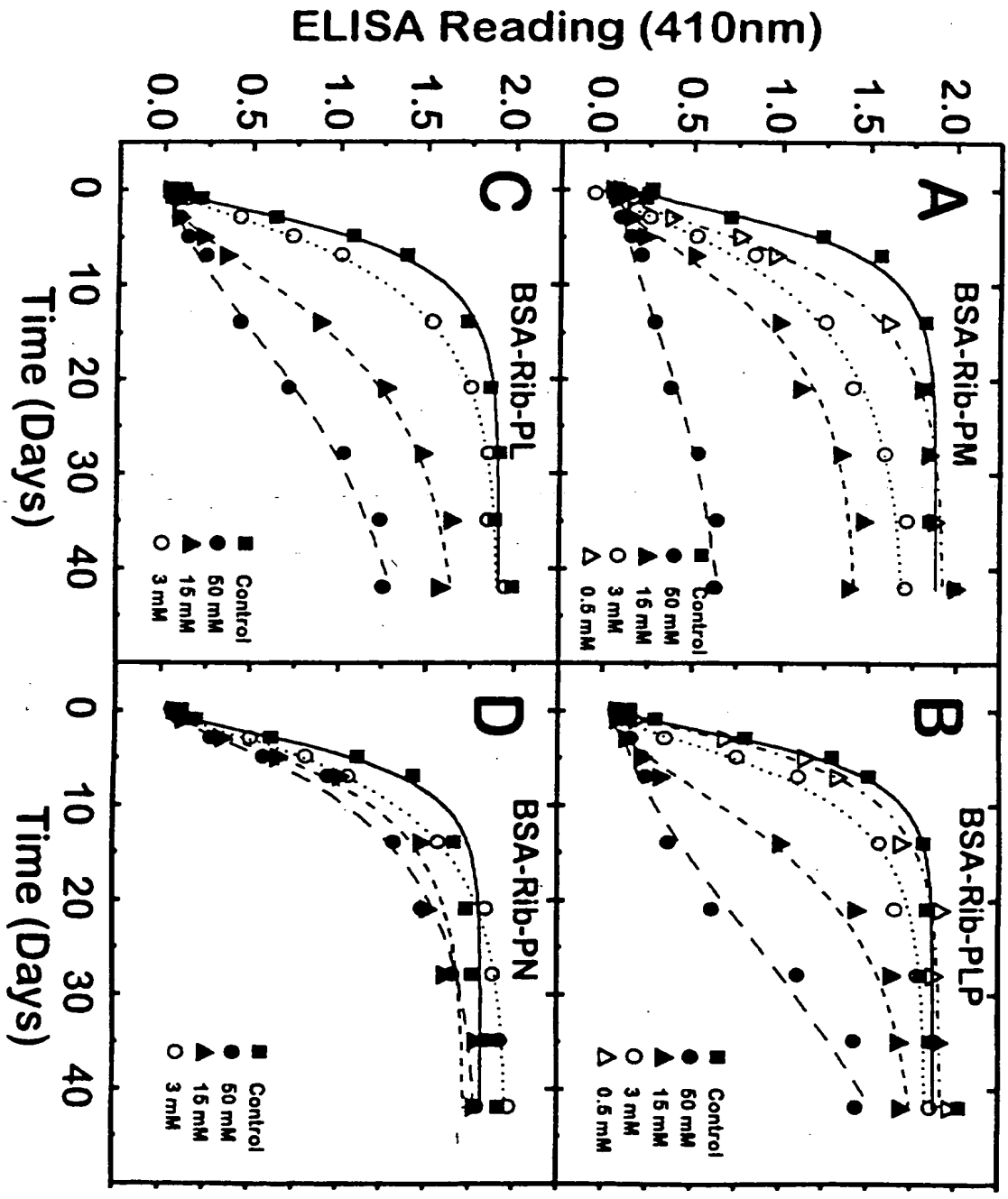

ELISA Reading (410nm)
0.0
0.5
1.0
1.5
2.0
A
BSA-Rib-PM
B
BSA-Rib-PLP
C
BSA-Rib-PL
D
BSA-Rib-PN
Control
50 mM
15 mM
3 mM
0.5 mM
0
10
20
30
40
Time (Days)

Fig.15

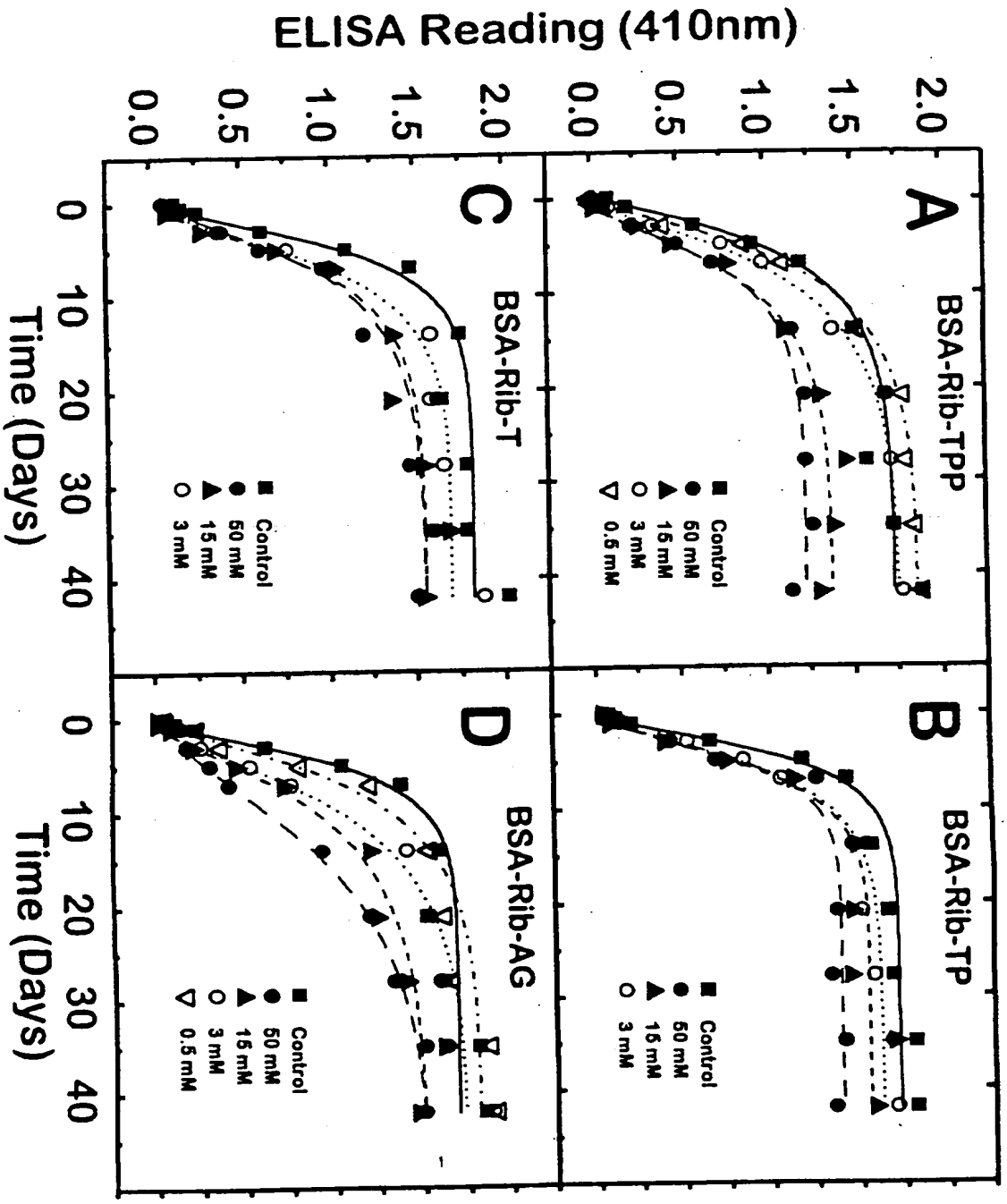

ELISA Reading (410nm)
0.0
0.5
1.0
1.5
2.0
A
BSA-Rib-TPP
Control
50 mM
15 mM
3 mM
0.5 mM
B
BSA-Rib-TP
Control
50 mM
15 mM
3 mM
C
BSA-Rib-T
Control
50 mM
15 mM
3 mM
D
BSA-Rib-AG
Control
50 mM
15 mM
3 mM
0.5 mM
0
10
20
30
40
Time (Days)

Fig. 16

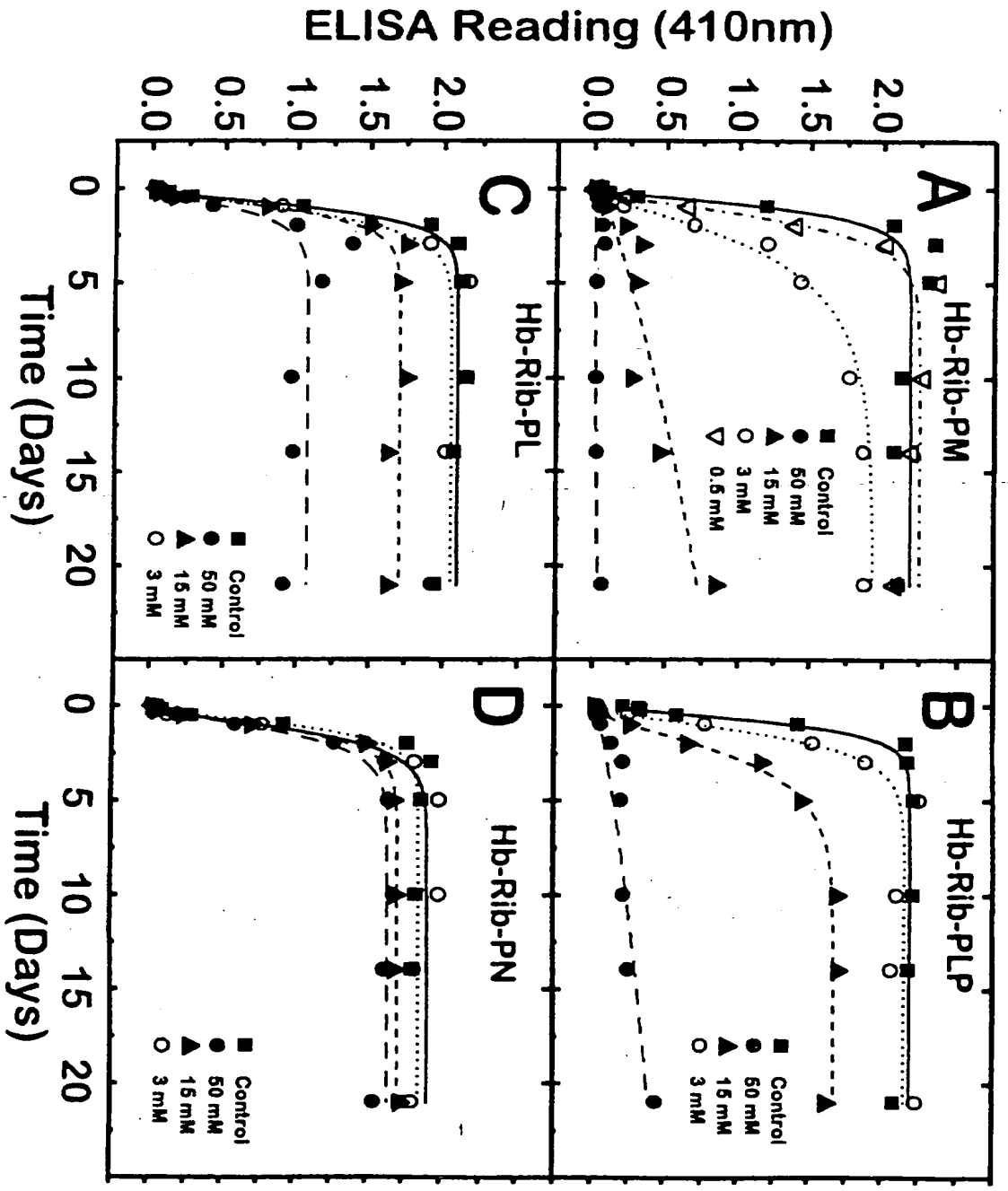

ELISA Reading (410nm)
0.0
0.5
1.0
1.5
2.0
A
Hb-Rib-PM
Control
50 mM
15 mM
3 mM
0.5 mM
B
Hb-Rib-PLP
Control
50 mM
15 mM
3 mM
C
Hb-Rib-PL
Control
50 mM
15 mM
3 mM
D
Hb-Rib-PN
Control
50 mM
15 mM
3 mM
0
5
10
15
20
Time (Days)

Fig. 17

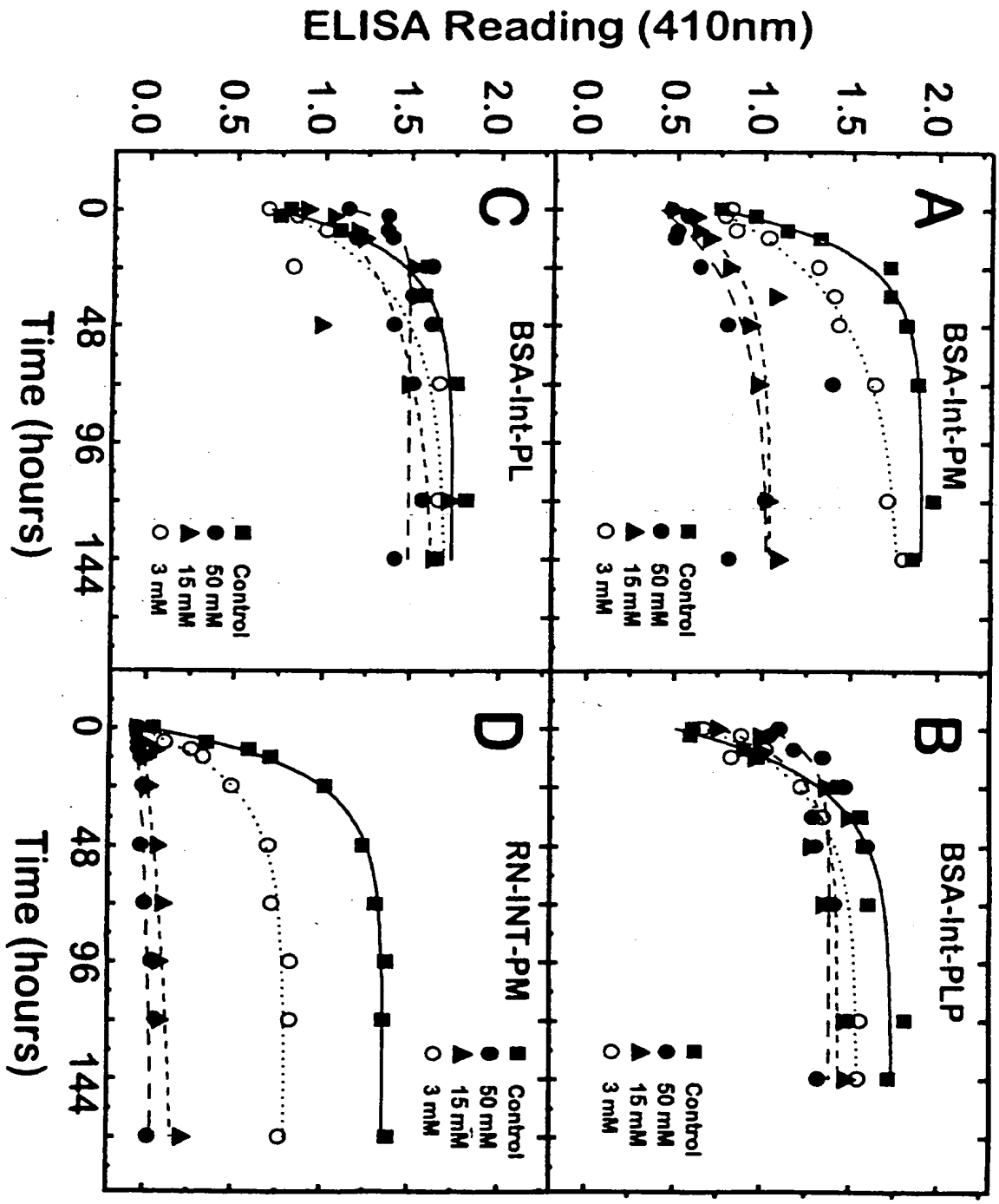

ELISA Reading (410nm)
A
BSA-Int-PM
B
BSA-Int-PLP
C
BSA-Int-PL
D
RN-INT-PM
Control
50 mM
15 mM
3 mM
Time (hours)
0.0
0.5
1.0
1.5
2.0
0
48
96
144

Fig. 18

Fig. 19

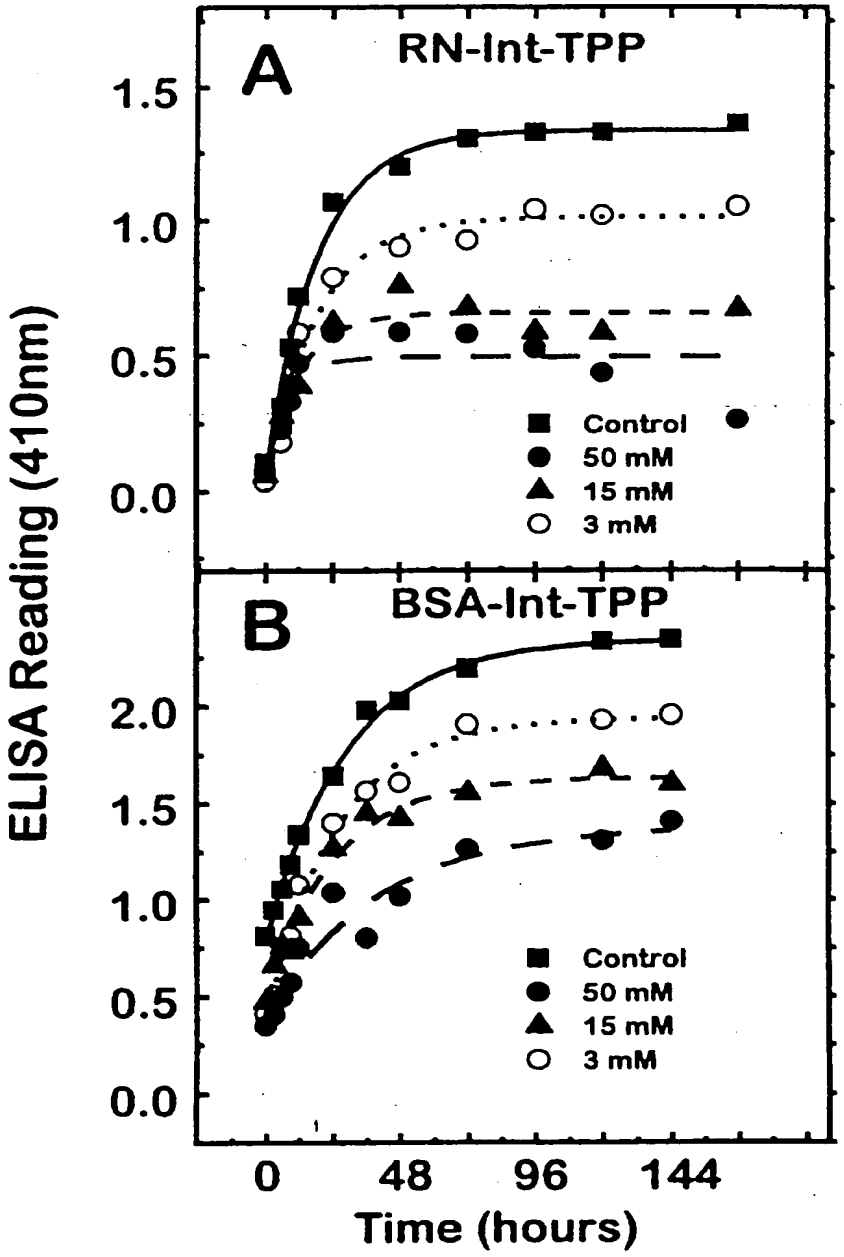
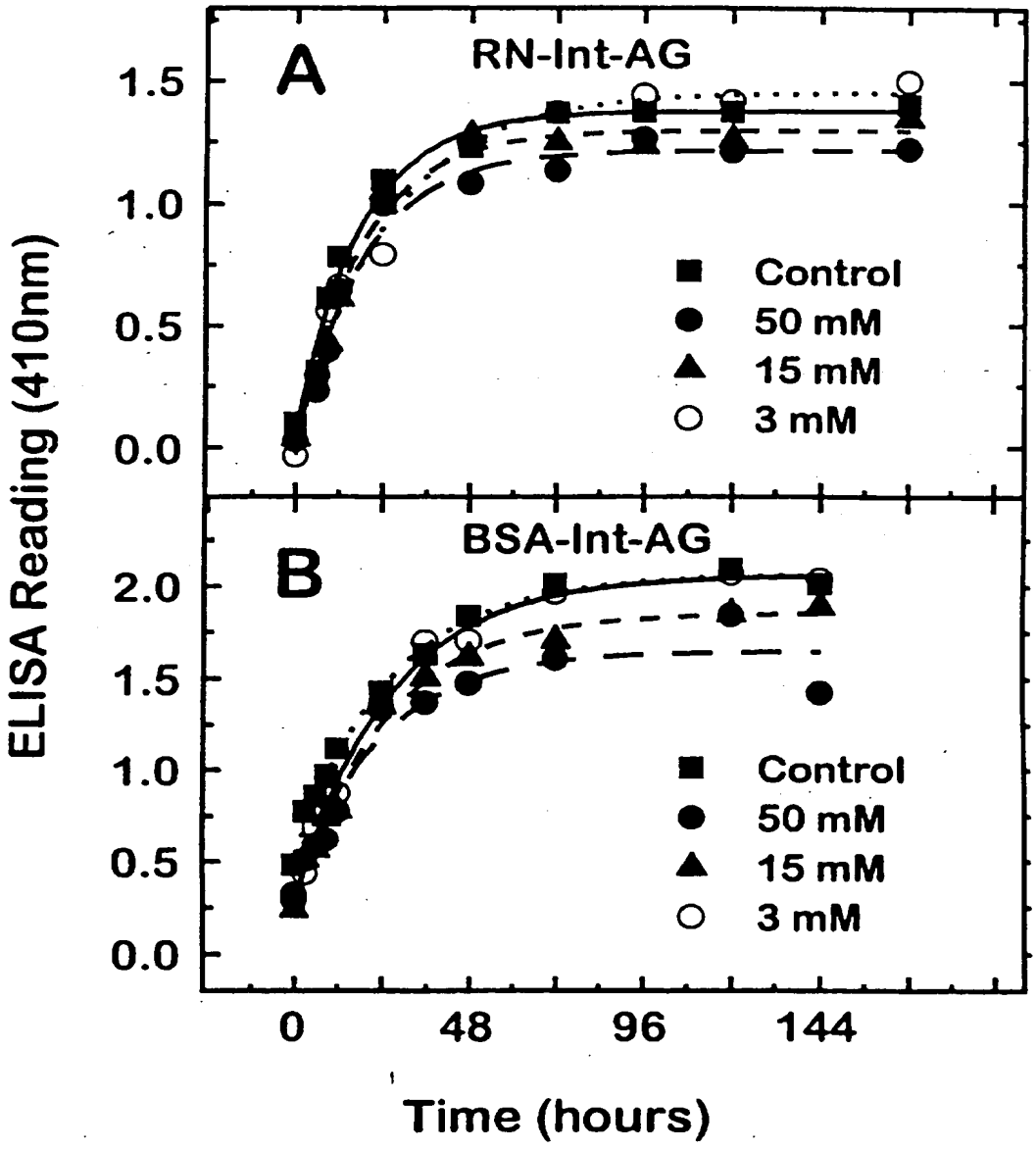

A
RN-Int-TPP
B
BSA-Int-TPP
ELISA Reading (410nm)
Time (hours)
Control
50 mM
15 mM
3 mM
0.0
0.5
1.0
1.5
2.0
0
48
96
144

Fig. 20


A
RN-Int-AG
B
BSA-Int-AG
Control
50 mM
15 mM
3 mM
ELISA Reading (410nm)
Time (hours)
0.0
0.5
1.0
1.5
2.0
0
48
96
144

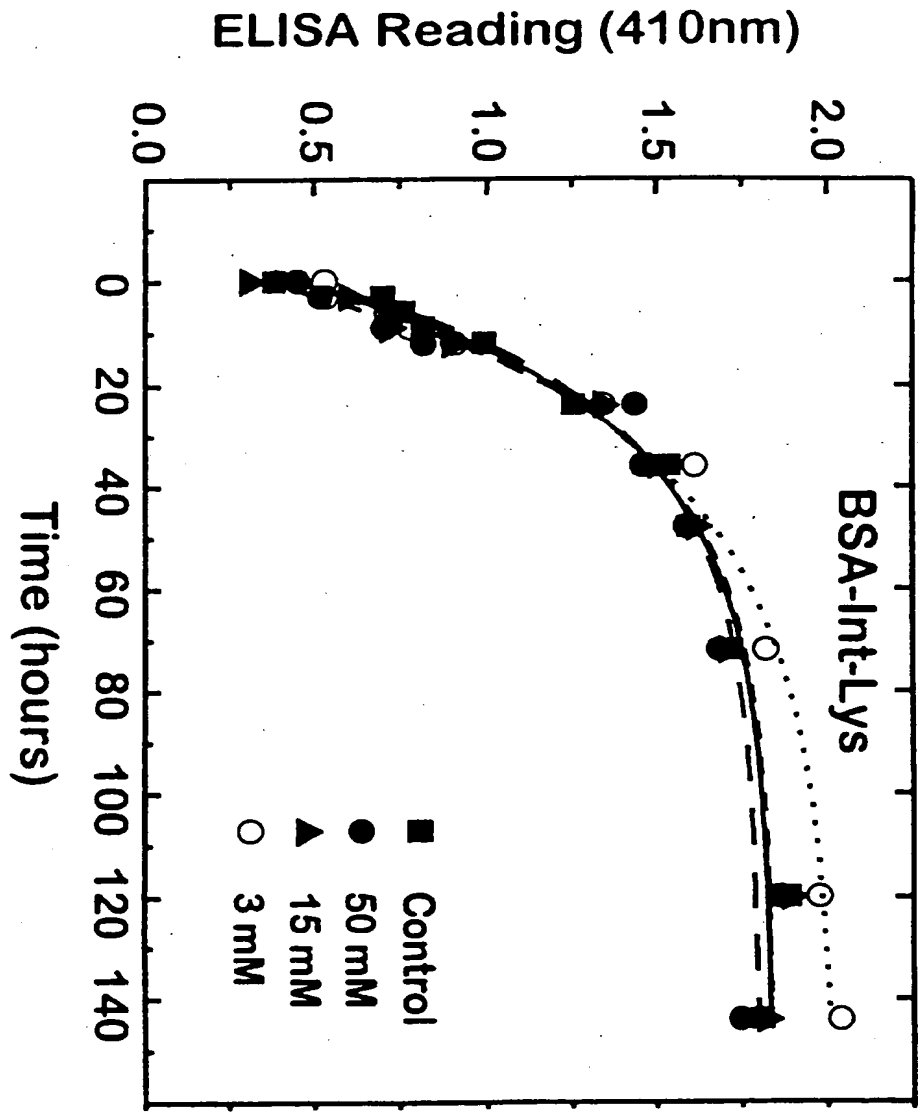

ELISA Reading (410nm)
0.0
0.5
1.0
1.5
2.0
0
20
40
60
80
100
120
140
Time (hours)
BSA-Int-Lys
Control
50 mM
15 mM
3 mM

Fig. 21

Fig. 22

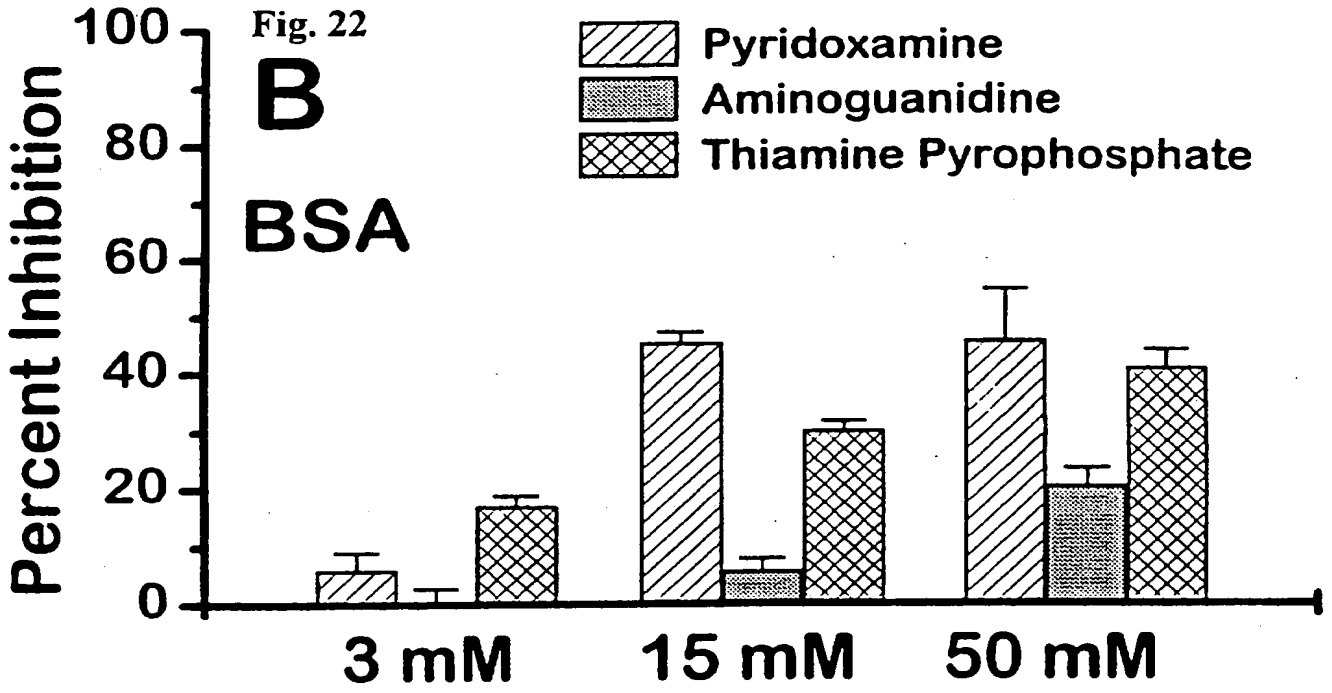
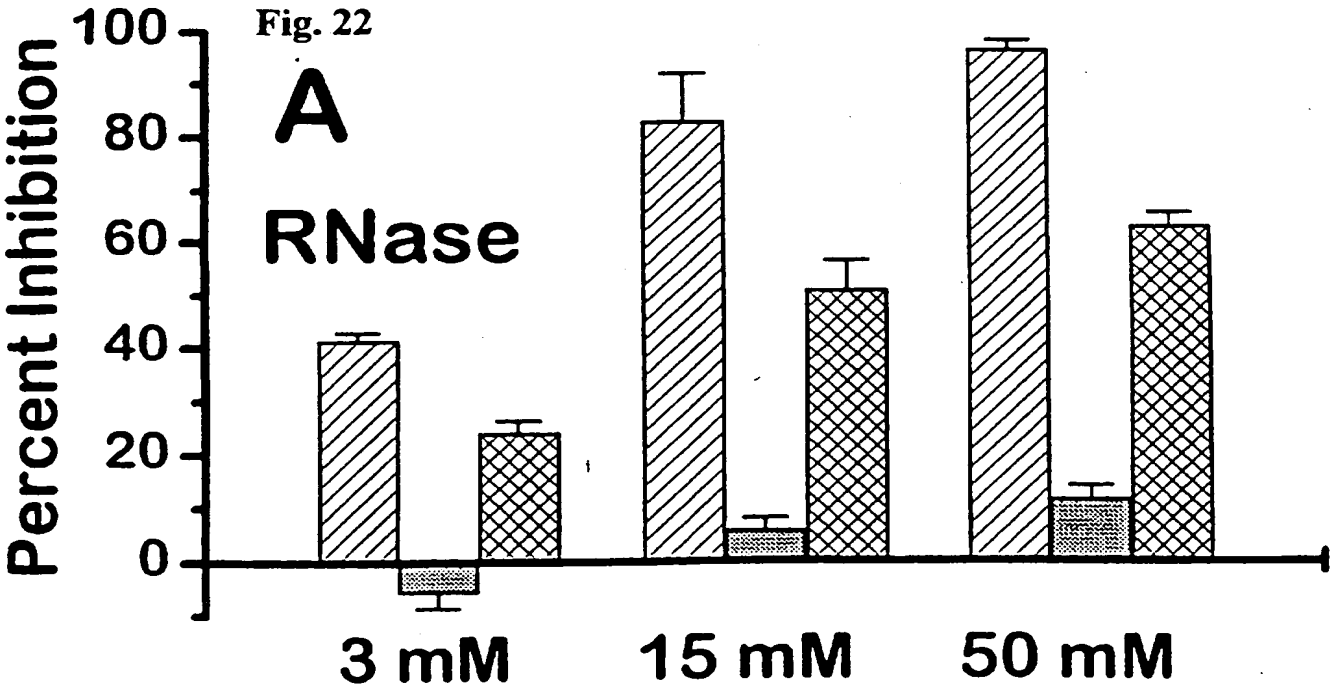

B
BSA
Pyridoxamine
Aminoguanidine
Thiamine Pyrophosphate
Percent Inhibition
100
80
60
40
20
0
3 mM
15 mM
50 mM

Fig. 22


A
RNase
Percent Inhibition
100
80
60
40
20
0
3 mM
15 mM
50 mM

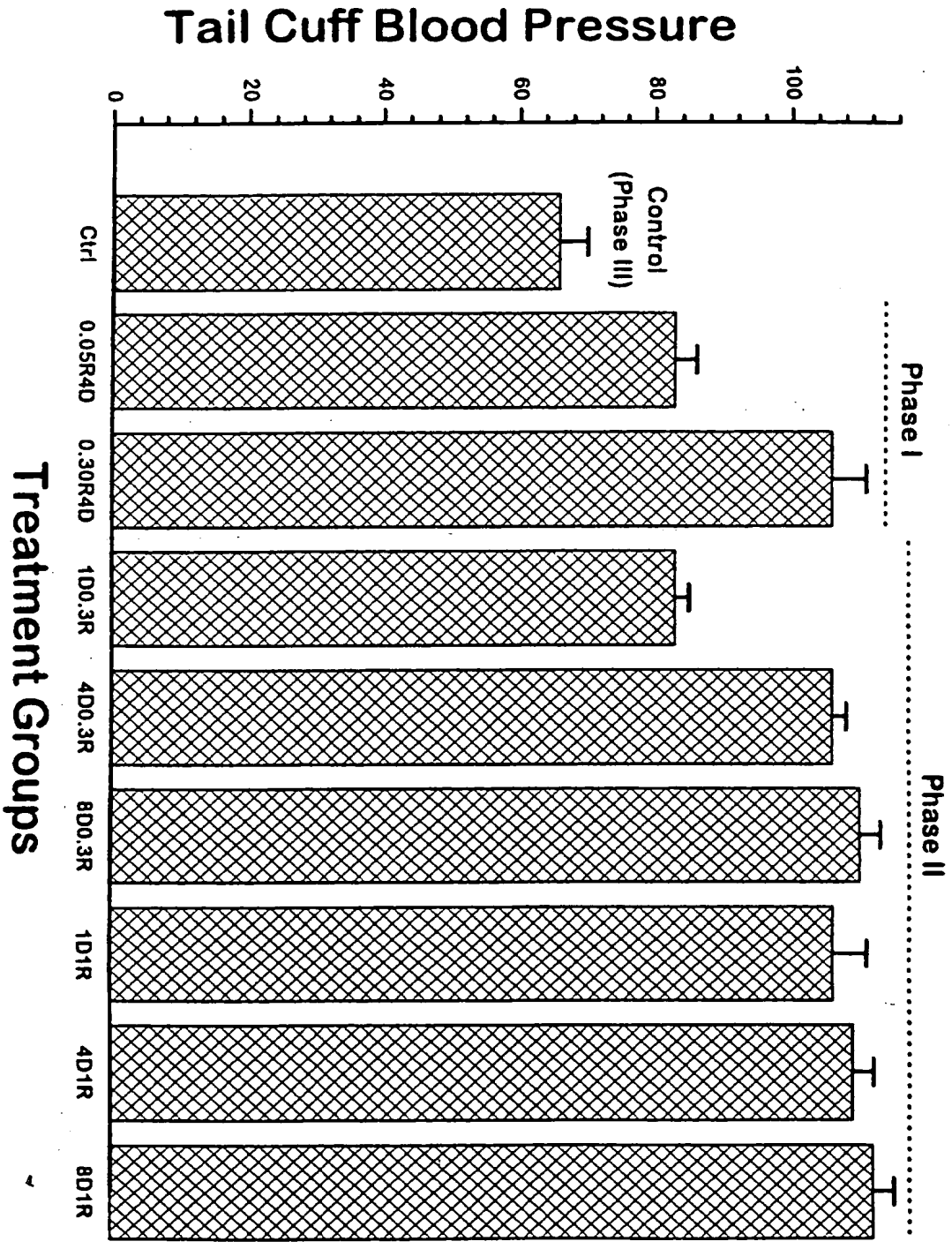

Tail Cuff Blood Pressure
0
20
40
60
80
100
Control (Phase III)
Phase I
Phase II
Ctrl
0.05R4D
0.30R4D
1D0.3R
4D0.3R
8D0.3R
1D1R
4D1R
8D1R
Treatment Groups

Fig.23

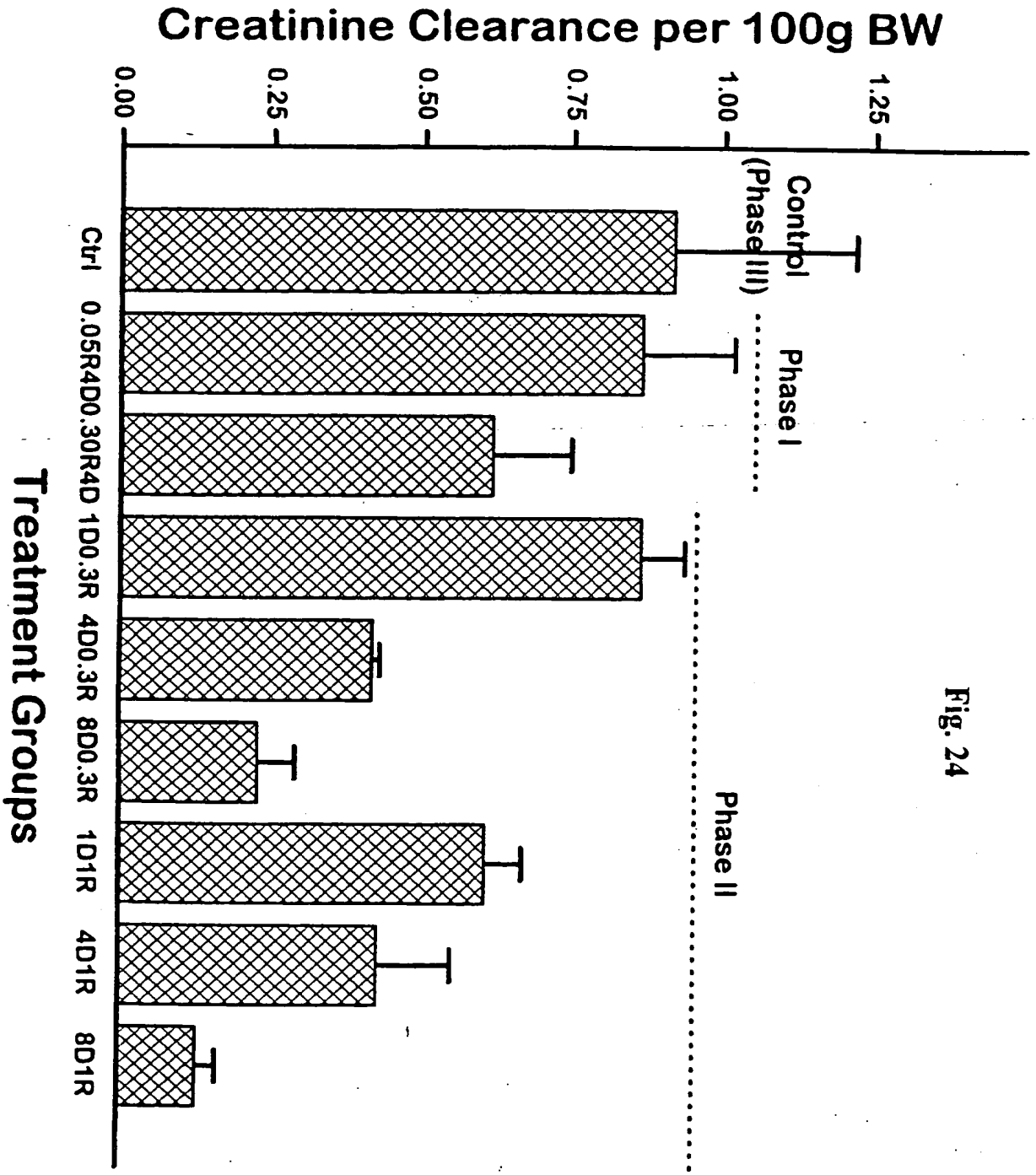

Creatinine Clearance per 100g BW
0.00
0.25
0.50
0.75
1.00
1.25
Control (Phase III)
Phase I
Phase II
Ctrl
0.05R4D
0.30R4D
1D0.3R
4D0.3R
8D0.3R
1D1R
4D1R
8D1R
Treatment Groups

Fig. 24

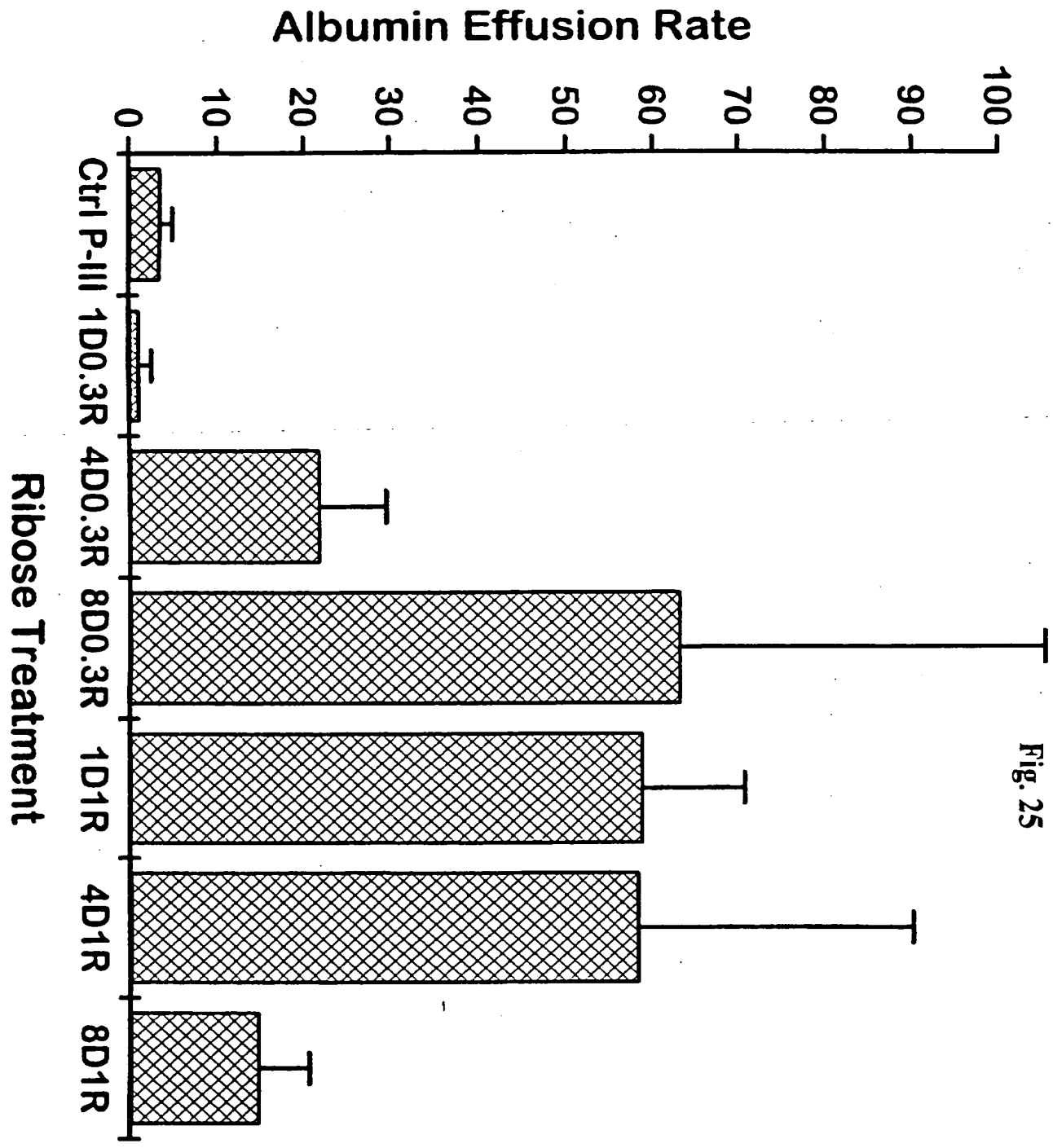

Albumin Effusion Rate
0
10
20
30
40
50
60
70
80
90
100
Ctrl
P-III
1D0.3R
4D0.3R
8D0.3R
1D1R
4D1R
8D1R
Ribose Treatment

Fig. 25

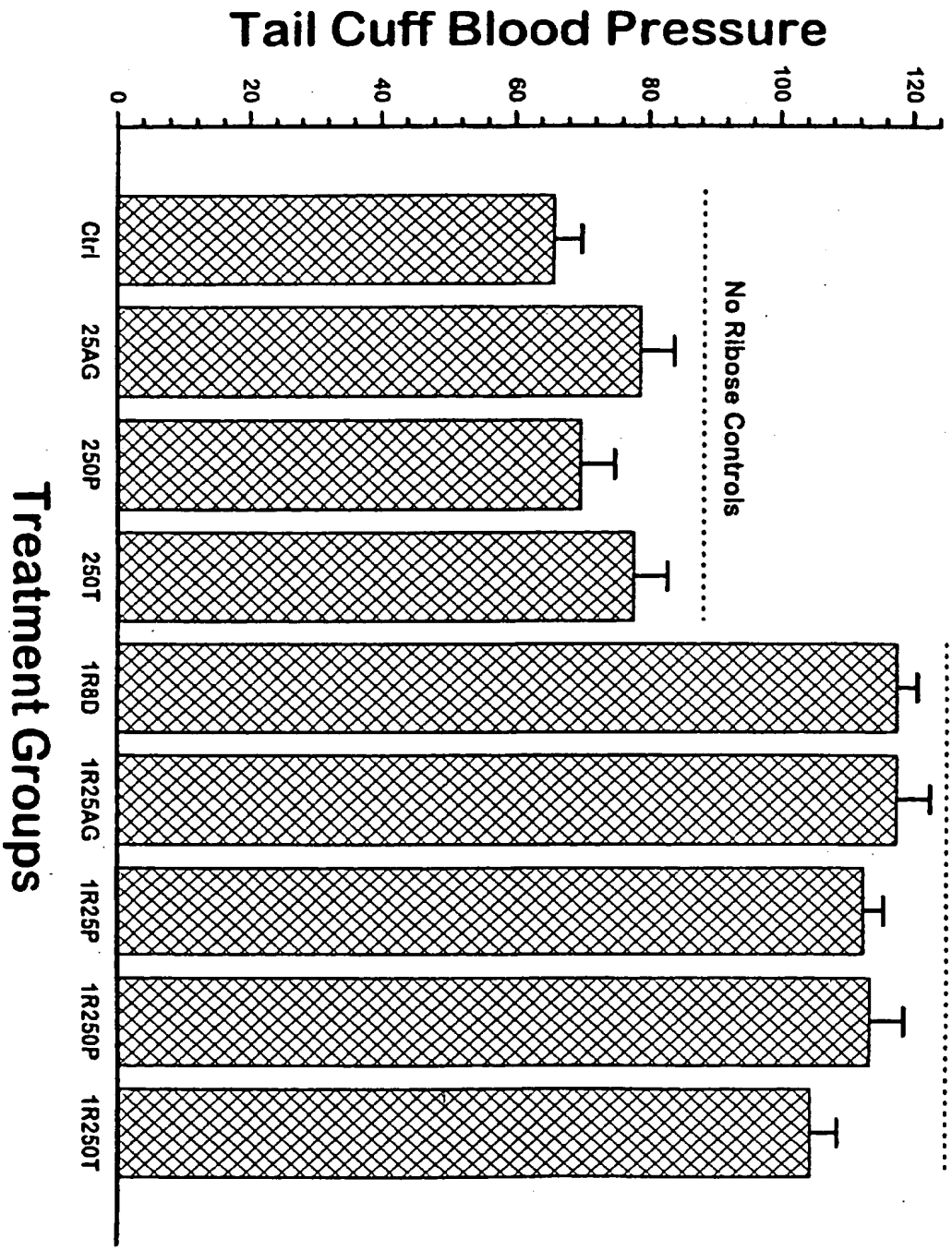

Tail Cuff Blood Pressure
0
20
40
60
80
100
120
No Ribose Controls
1 M Ribose
Ctrl
25AG
250P
250T
1R8D
1R25AG
1R25P
1R250P
1R250T
Treatment Groups

Fig. 26

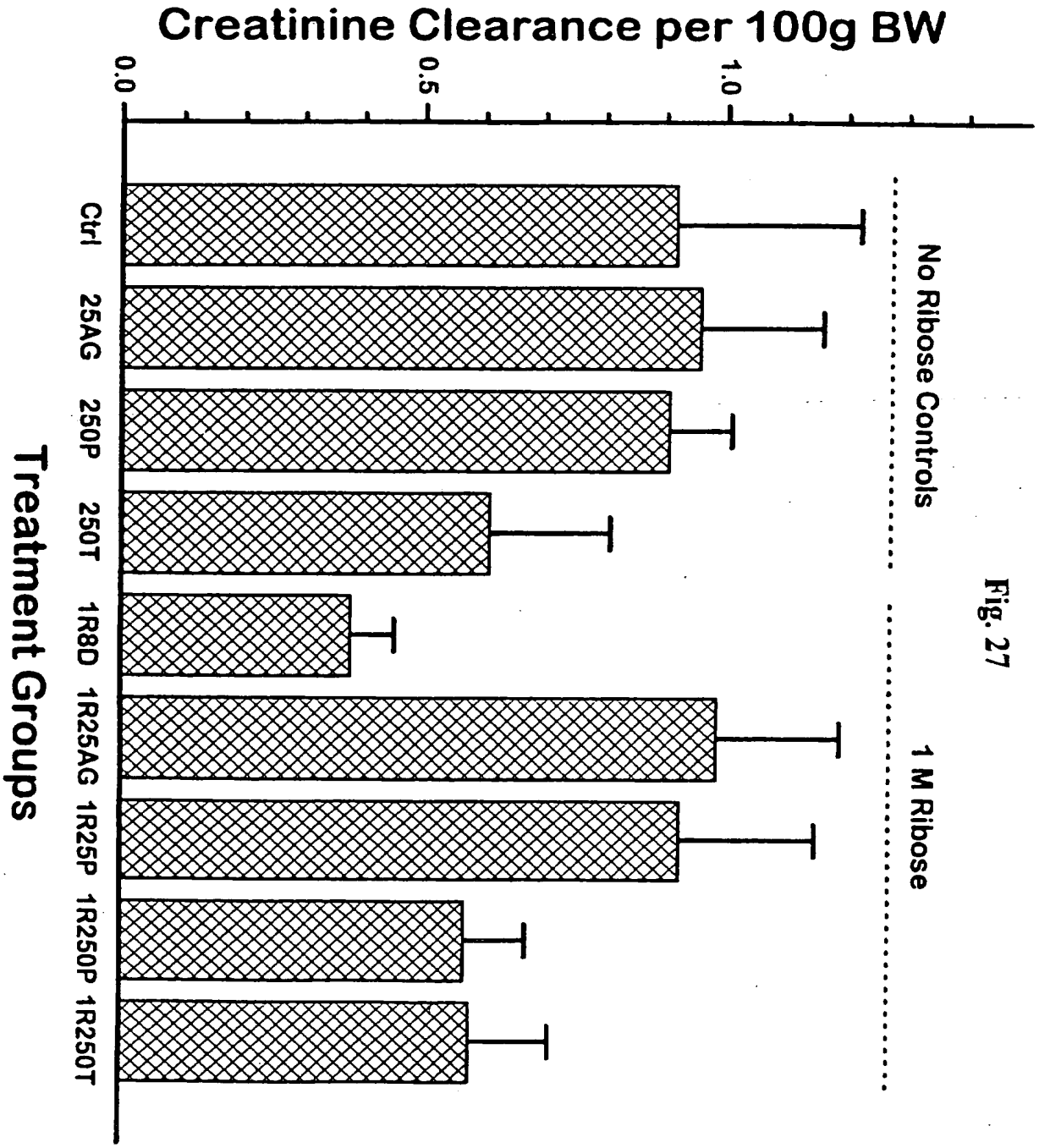

Creatinine Clearance per 100g BW
0.0
0.5
1.0
Ctrl
25AG
250P
250T
1R8D
1R25AG
1R25P
1R250P
1R250T
Treatment Groups
No Ribose Controls
1 M Ribose

Fig. 27

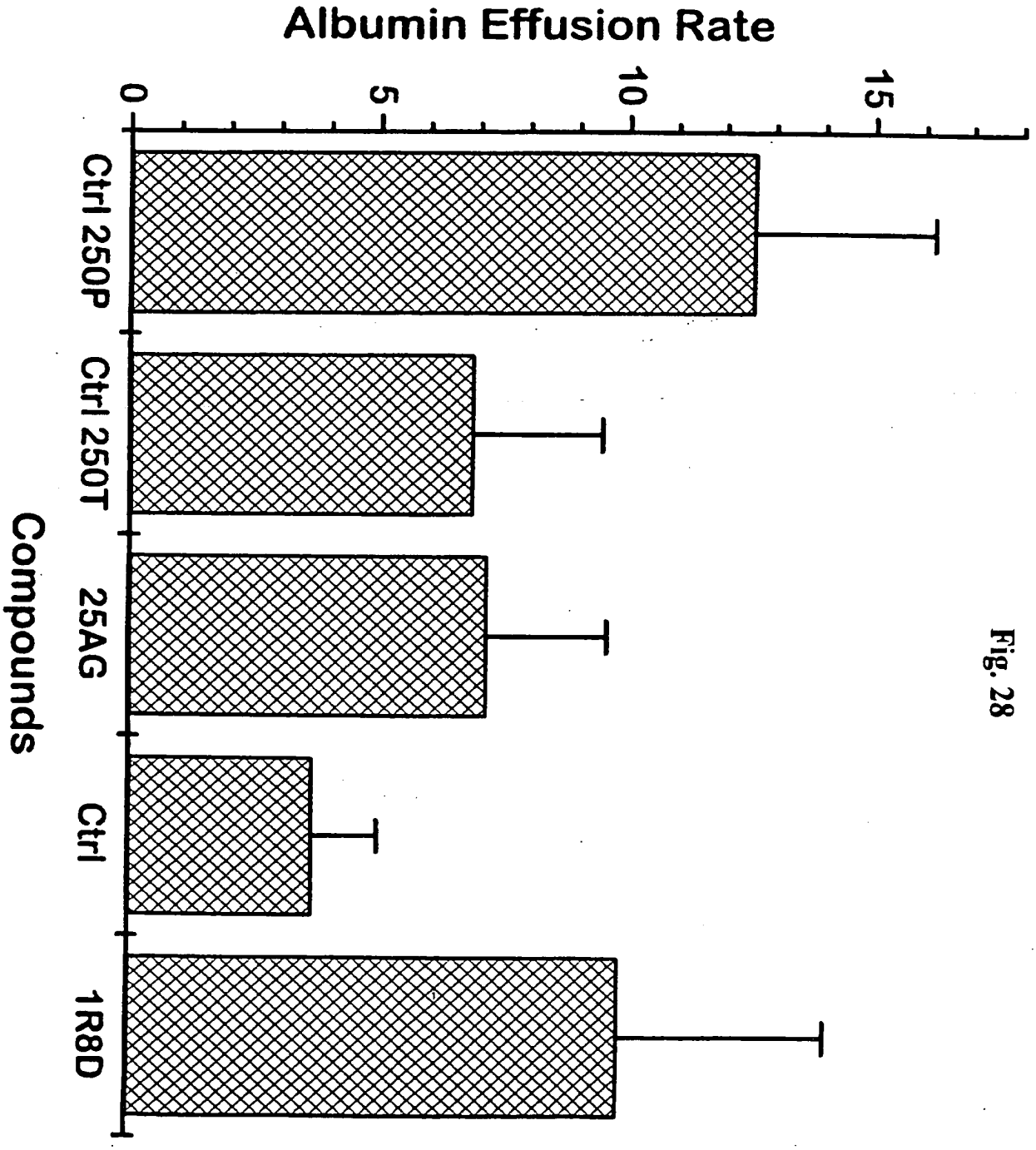

Albumin Effusion Rate
0
5
10
15
Ctrl 250P
Ctrl 250T
25AG
Ctrl
1R8D
Compounds

Fig. 28

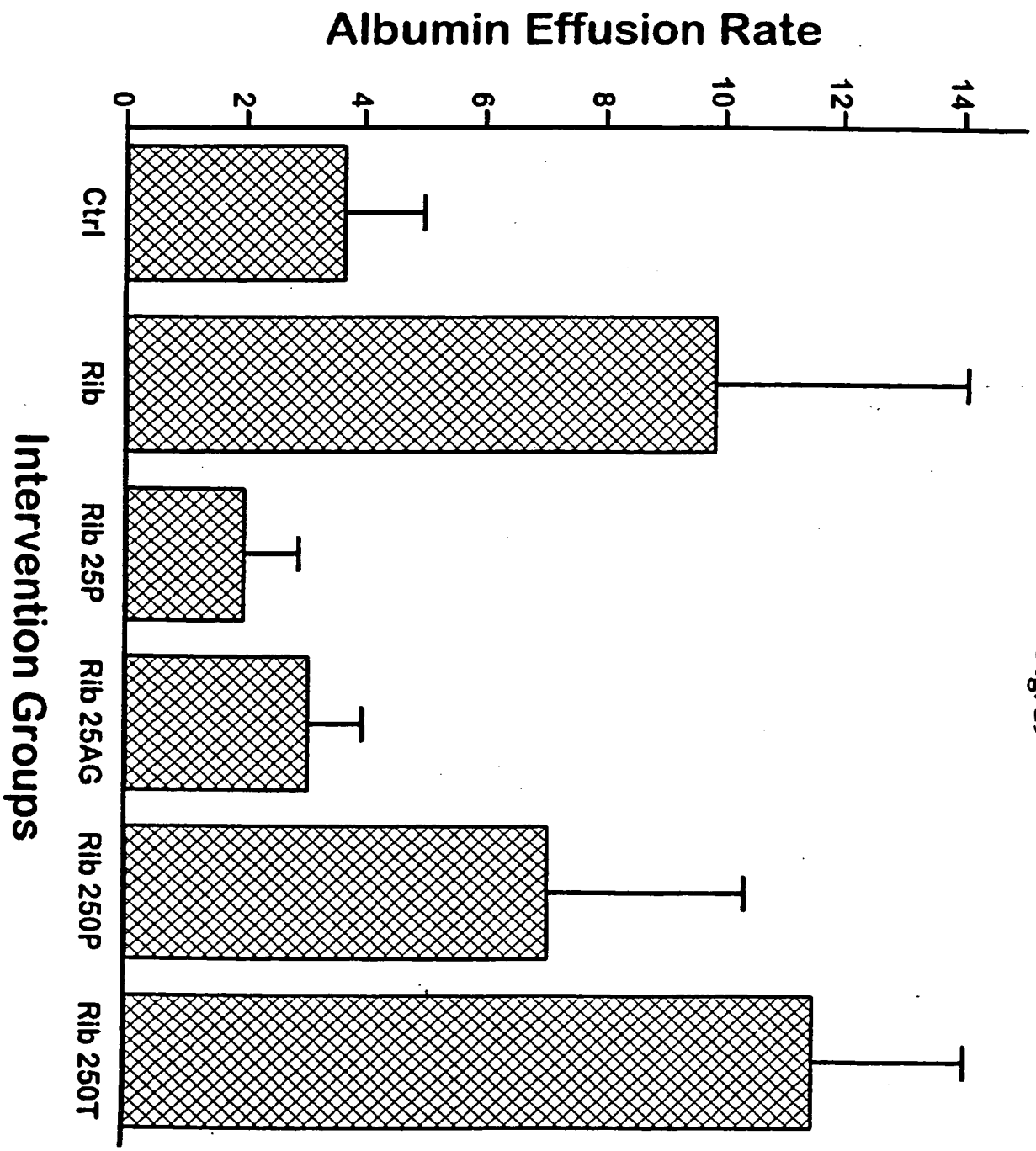
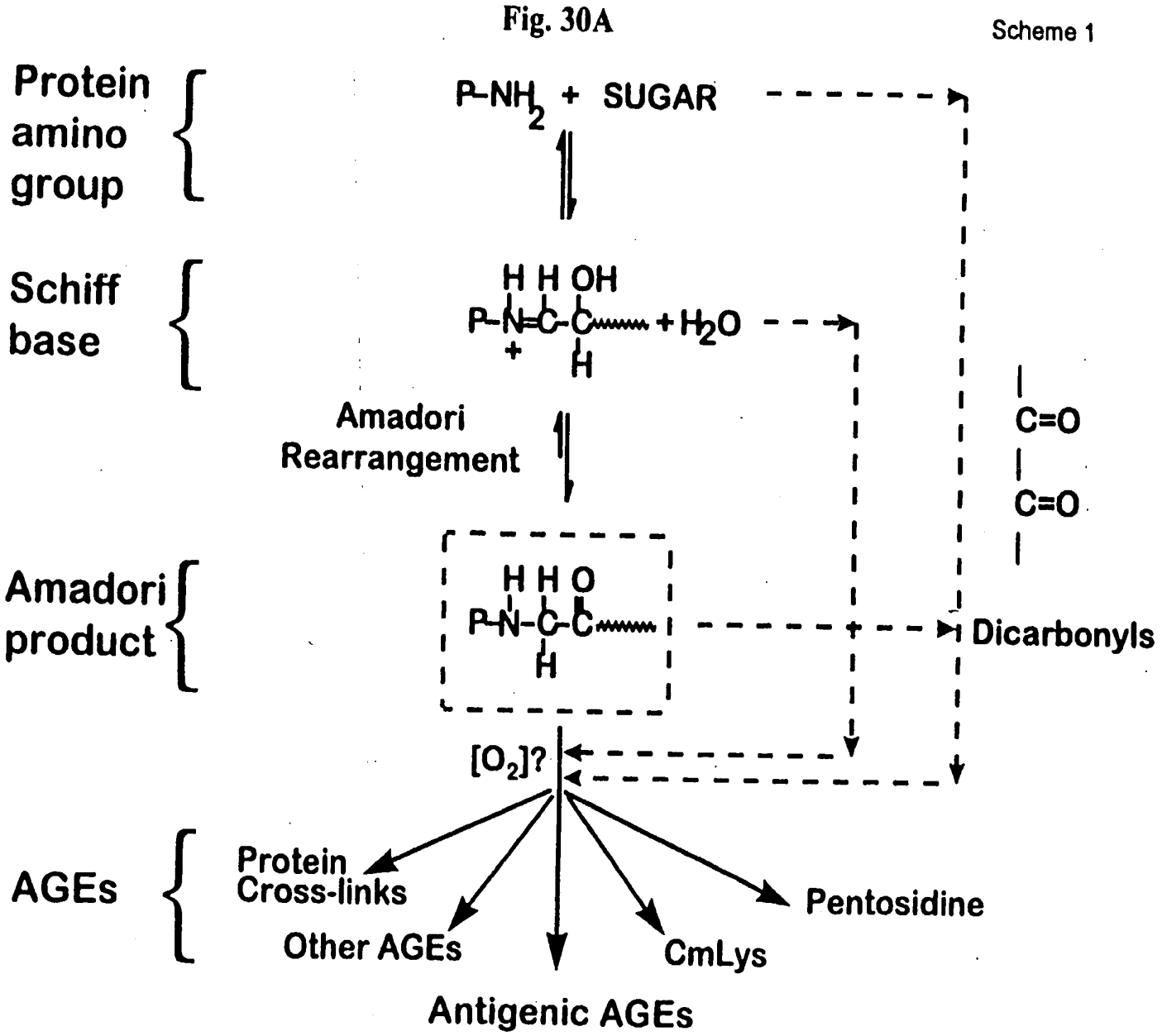

Albumin Effusion Rate
0
2
4
6
8
10
12
14
Ctrl
Rib
Rib 25P
Rib 25AG
Rib 250P
Rib 250T
Intervention Groups

Fig. 29

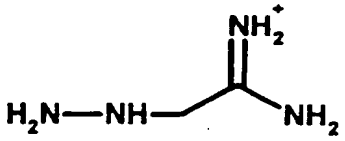
Fig. 30A


Scheme 1
Protein amino group
P–NH2 + SUGAR
Schiff base
H H OH
P–N=C–C + H2O
+ H
Amadori Rearrangement
C=O
C=O
Amadori product
H H O
P–N–C–C
H
Dicarbonyls
[O2]?
AGEs
Protein Cross-links
Other AGEs
CmLys
Pentosidine
Antigenic AGEs

Scheme 2

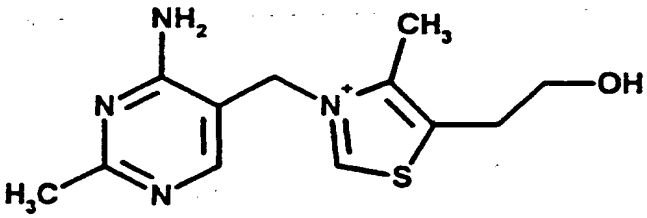
Fig. 30B

Aminoguanidine

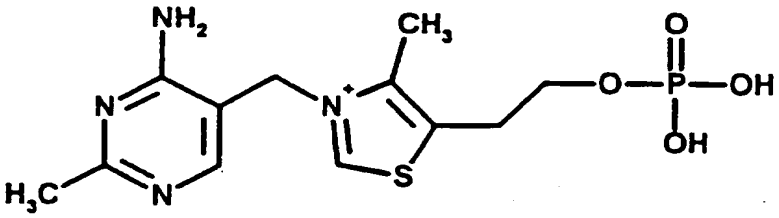


Scheme 3

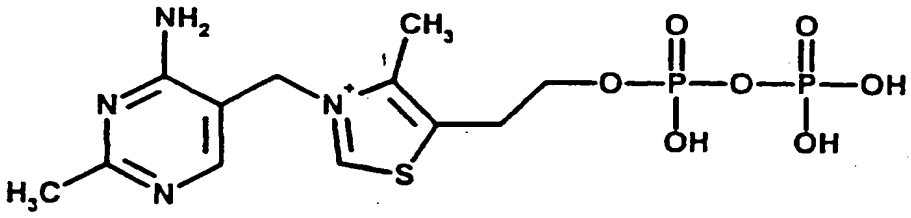
Fig. 30C



Thiamine



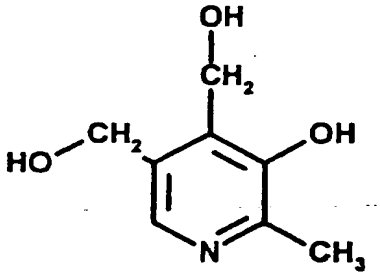
Thiamine-5'-Phosphate



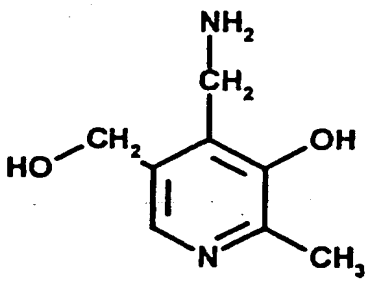
Thiamine Pyrophosphate

Scheme 4

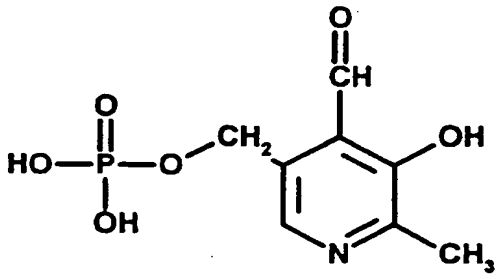
Fig. 30D



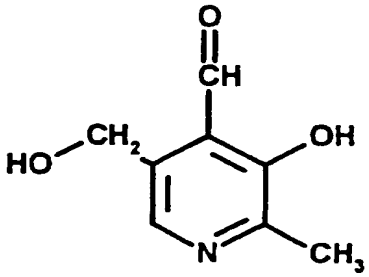
Pyridoxine



Pyridoxamine



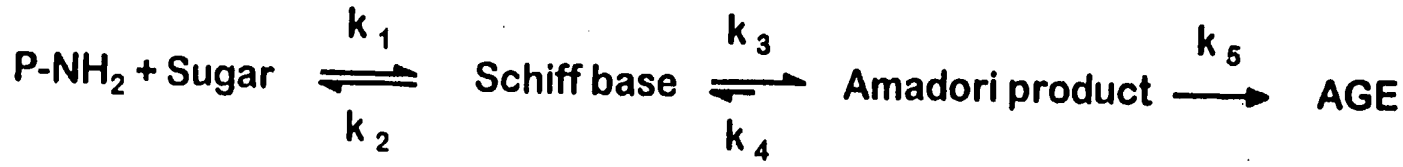
Pyridoxal-5'-Phosphate



Pyridoxal

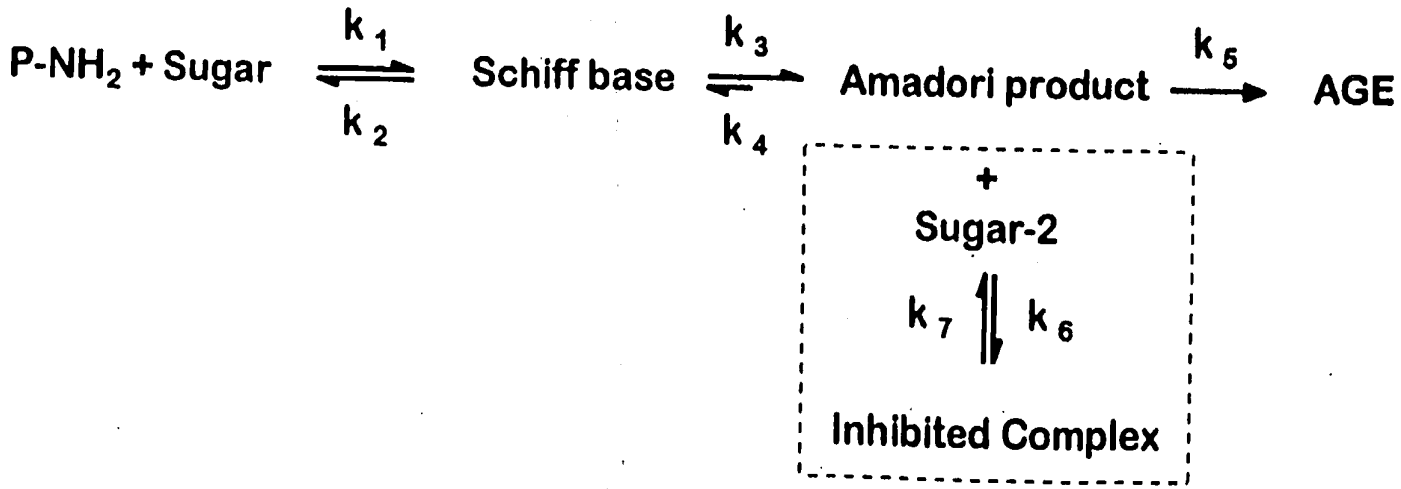
Scheme 5

Fig. 30E



Scheme 6

Fig. 30F



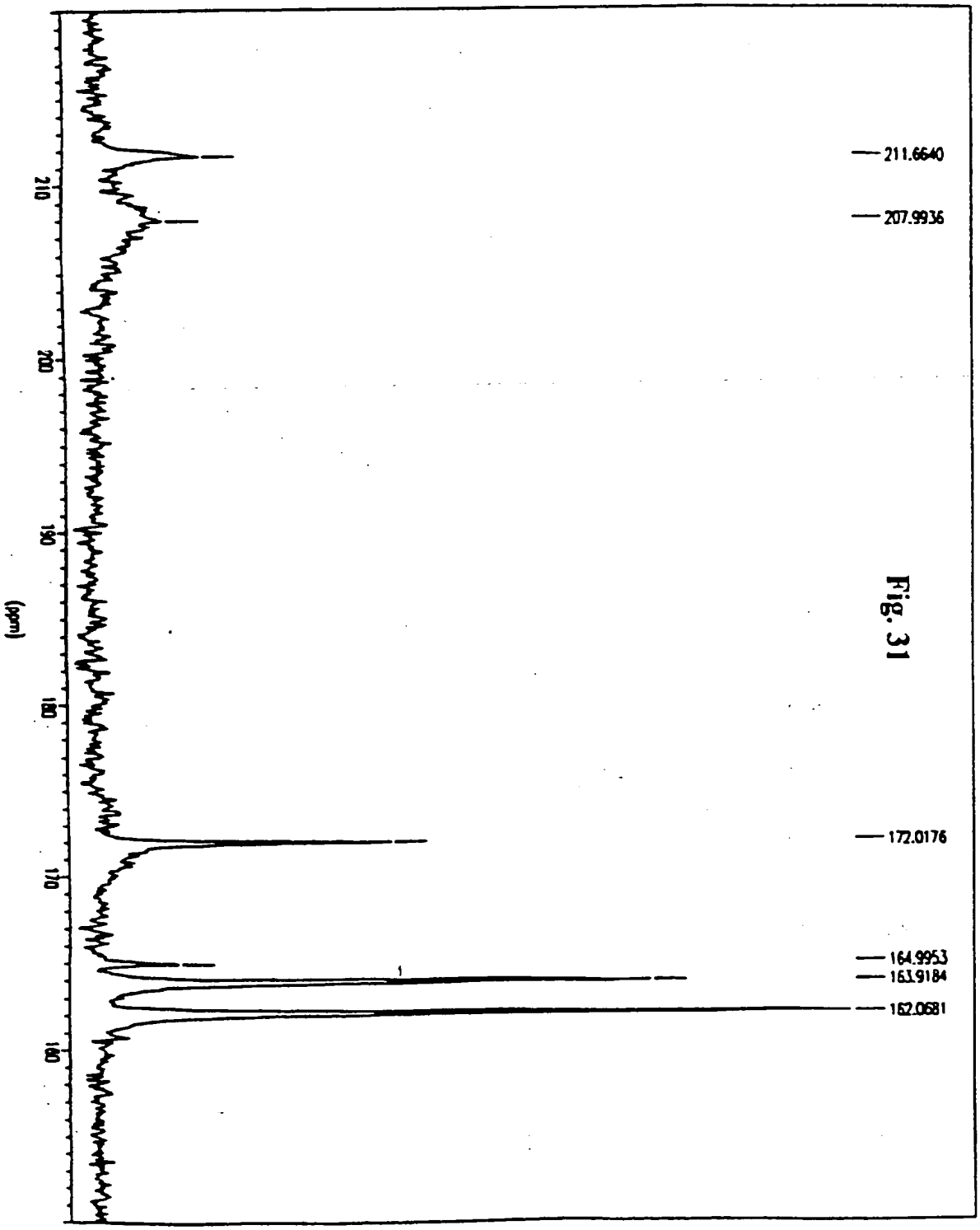

211.6640
207.9936
172.0176
164.9953
163.9184
162.0681
210
200
190
180
170
160
(ppm)

Fig. 31

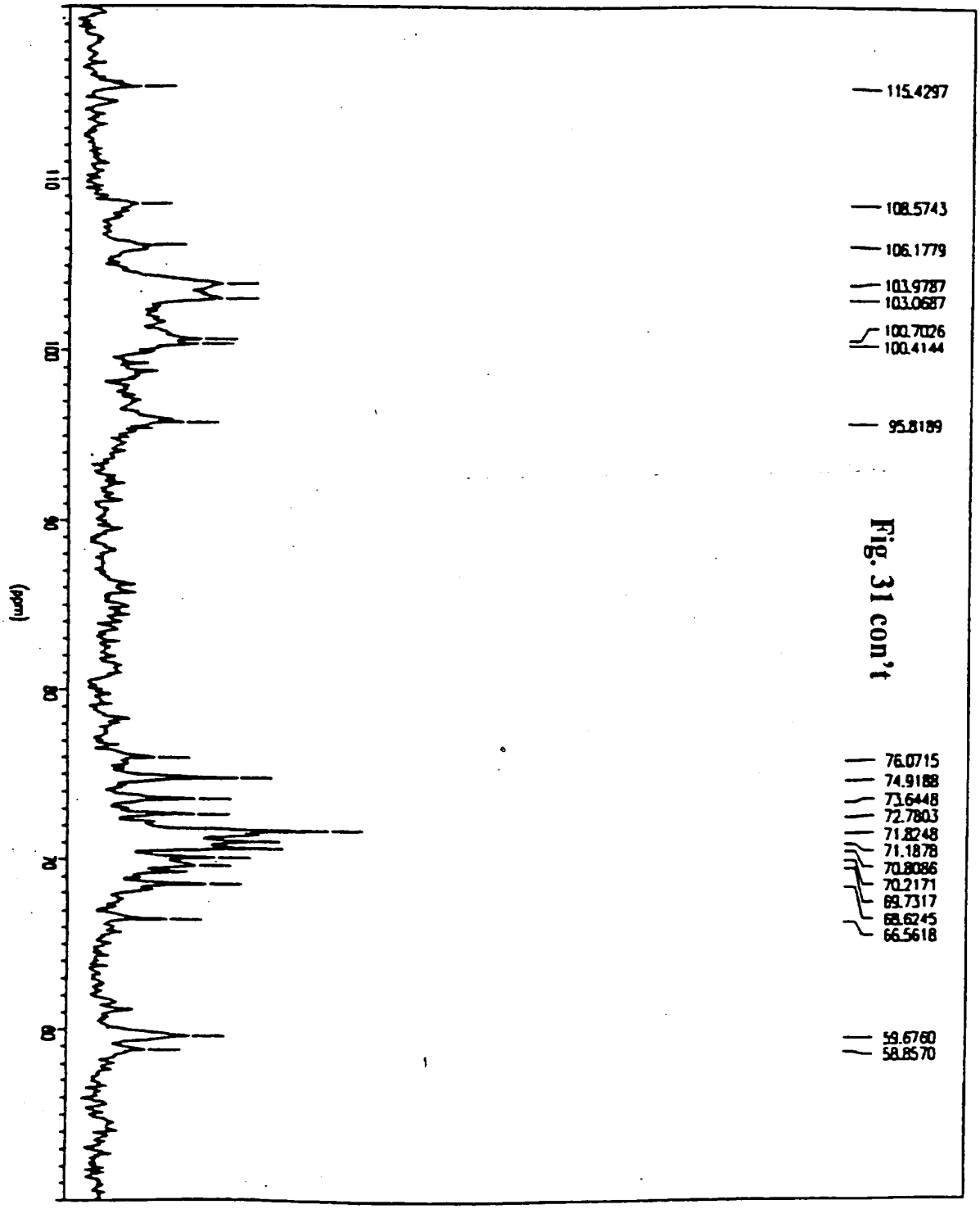

115.4297
108.5743
106.1779
103.9787
103.0687
100.7026
100.4144
95.8189
76.0715
74.9188
73.6448
72.7803
71.8248
71.1878
70.8086
70.2171
69.7317
68.6245
66.5618
58.6760
58.8570
110
100
90
80
70
60
(ppm)

Fig. 31 con't

INTERNATIONAL SEARCH REPORT

Interna·nal Application No
PCT/US 96/14544

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/425 A61K31/44 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 02216 A (THE ROCKEFELLER UNIVERSITY) 20 February 1992 see the whole document ---	1-19
X,P	S BUDAVARI ET AL., EDS. : "The Merck Index" 1996 , MERCK & CO, INC. , WHITEHOUSE STATION, USA XP002024847 408196 see page 1586, no. 9431 ---	19
X,P	S BUDAVARI ET AL., EDS.: "The Merck Manual" 1996 , MERCK & CO., INC. , WHITEHOUSE STATION, USA XP002024848 408196 see page 1372, no. 8164 ---	19

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 February 1997	26.02.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Masturzo, P

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INTERNATIONAL SEARCH REPORT

Interna[illegible]nal Application No
PCT/US 96/14544

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	BIOCHEMISTRY, vol. 35, no. 15, 16 April 1996, EASTON, PA US, pages 4645-4654, XP002024844 R G KHALIFAH ET AL.: "Kinetics of nonenzymatic glycation of ribonuclease A leading to advanced glycation end products..." see the whole document	1-19
P,X	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 220, no. 1, 1996, ORLANDO, FL US, pages 113113-119, XP002024845 A A BOOTH ET AL: "Thiamine pyrophosphate and pyridoxamine inhibit the formation of advanced glycation end products..." see the whole document	1-19
X,P	S BUDAVARI ET AL., EDS.: "The Merck Index" 1996 , MERCK & CO. WHITEHOUSE STATION, USA XP002024849 408196 see page 1411, no. 8371	19
A	JOURNAL OF IMMUNOLOGICAL METHODS, vol. 140, no. 1, 1991, NEW YORK US, pages 119-125, XP002024846 H NAKAYAMA ET AL.: "Characteirzation of antibodies to advanced glycosylation end products on proteins" see the whole document	1-19
A	CHEMICAL ABSTRACTS, vol. 116, no. 19, 11 May 1992 Columbus, Ohio, US; abstract no. 190396, P ODETTI ET AL.: "Chromatographic quantitation of plasma and erythrocyte pentosidine in diabetic and uremic subjects" XP002024850 see abstract & DIABETES, vol. 41, no. 2, 1992, pages 153-159, cited in the application	1-19

3

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

:national application No.

PCT/US 96/ 14544

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1-5, 12-17
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1-5(at least partially) and 12-17 refer to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compounds.

2. [X] Claims Nos.: 2
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
Remark: Fructose is mentioned Twice in claim 2.

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

ormation on patent family members

Internat nal Application No
PCT/US 96/14544

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9202216	20-02-92	AU-A-	8449591	02-03-92
		US-A-	5476849	19-12-95
		US-A-	5514676	07-05-96
		US-A-	5272176	21-12-93